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The Pathogenesis and Genetic Diversity of Rodent Torque Teno Virus

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Thesis presented for the degree of Doctor of Philosophy

The University of Edinburgh

2013

Declaration

I hereby declare that this thesis is of my own composition, and that it contains no material previously submitted for the award of any other degree. The work reported in this thesis has been executed by myself, except where due acknowledgement is made in the text.

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Abstract

Torque teno virus (TTV) is a single stranded circular DNA virus and, despite its widespread nature in the human population, its pathogenesis is still unknown. Factors complicating TTV research include its huge genetic diversity, difficulties identifying an uninfected control population, the lack of a small animal model and lack of a good cell culture system for viral propagation. Recently we have identified a TTV homologue (RoTTV) in wild rodents. RoTTV was frequently observed in wood mice and field voles. RoTTV infections were also found in bank voles but not in *Mus musculus* populations. Analysis of complete genome sequencing shows that several genetic variants are found in wild rodent population with two distinct species containing several diverse genotypes. Furthermore, multiple variants were present in single individuals, consistent with infection patterns seen in humans.

RoTTV transcripts in infected wild wood mice have also been detected and fully sequenced. Predicted protein coding regions from these transcripts have been expressed in cell culture and show the different expression patterns. Using cloned genomic DNA it has also been possible to observe the transcription from the virus *in vitro* and it was shown Ro TTV viral titer in the supernatant of culture fluid increased. In addition, Ro TTV propagation was observed by using the supernatant of culture fluid. Using cloned genomic DNA and the culture supernatant, an *in vivo* model system in naïve laboratory wood mice was developed.

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Contents

Abstract	ii
Acknowledgements	iii
Contents	iv
Figures	x
Tables	xii
Abbreviations	xiii

Chapter I: INTRODUCTION	1
1.1 <i>Torque teno virus</i> (TTV) and related <i>Anellovirus</i>	2
1.1.1. TTV discovery	3
1.1.2. TTMV discovery	4
1.1.3. TTMDV discovery and Small anellovirus (SAV) Discovery	4
1.1.4. Animal TTV discovery	5
1.1.4.1. TTV infection in Nonhuman Primates	7
1.1.4.2. TTV infection in farm animal	8
1.2. Genetic diversity	8
1.3. Epidemiology/Transmission	13
1.3.1. Epidemiology	13
1.3.2. Transmission	16
1.3.2.1. Vertical transmission	16
1.3.2.2. Horizontal transmission	17
1.4. TTV structure and coding	19
1.4.1. Genome structure	19
1.4.2. Transcripts	21
1.4.3. Proteins	25
1.4.4. Genomic structure and predicted proteins of TTMV, TTMDV and SAV	28
1.5. Replication and Pathogenesis	29
1.5.1. Replication mechanism	29
1.5.2. Replication site and Pathogenicity of TTV	31
1.5.2.1. Virus replication in liver and hepatic disease	31

1.5.2.2. Virus replication in respiratory tract and pulmonary disease	35
1.5.2.3. Cancer	36
1.5.2.4. Autoimmune disorder	38
1.5.2.5. Virus replication in bone marrow cells and Haematological disorder	40
1.5.2.6. Co-infection with other pathogen	41
1.5.2.7. Immunocompromised patients	41
1.6. Immunology of TTV	42
1.6.1. Innate immunity	42
1.6.2. Adaptive immunity	43
1.6.3. Cell-mediated immunity	45
1.7. Model of TTV infection	46
1.7.1. Cell culture system of TTV	46
1.7.2. Animal model of TTV	47
1.8. Aims of project	52
 Chapter II. MATERIALS AND METHODS.....	 53
2.1. Tissue samples	54
2.2. DNA extraction.....	54
2.2.1. DNA extraction	54
2.2.2. Quantification of DNA.....	55
2.3. RNA Methods	55
2.3.1. Tissue collection and store condition	55
2.3.2. RNA extraction	56
2.3.3 DNase treatment of RNA using Turbo DNA-free™Procedure.....	56
2.3.4. Measurement of quality and quantity of RNA by using Agilent RNA 6000 Nano	57
2.4. Reverse transcription of RNA	57
2.5. Polymerase chain reaction (PCR)	57
2.5.1. Standard PCR/primer design.....	57
2.5.1.1. pan-TTV primers used for the screening	58
2.5.1.2. Screening PCR using genotype specific primer (ORF1primer) in wild rodent	58
2.5.2. Full genome-length PCR.....	58
2.5.3 Agarose gel electrophoresis	63

2.6. RFLP (Restriction Fragment Length Polymorphism) for identification of species	60
2.7. PCR product column purification and gel extraction	61
2.7.1. MinElute® PCR purification kit	61
2.7.2. QIAquick gel extraction kit	61
2.8. Sequence	61
2.8.1. Nucleotide sequence	62
2.8.2. Protein coding sequence	62
2.9. Molecular cloning	62
2.9.1. Ligation	62
2.9.2. Transformation of competent cells with plasmid DNA	63
2.9.3. Colony screening PCR	63
2.9.4. Plasmid DNA extraction	63
2.9.4.1. Small scale isolation of plasmid DNA using a QIAprep Miniprep kit QIAGEN	63
2.9.4.2. Large scale isolation of plasmid DNA using a Endo free maxiprep	64
2.9.5. T4 ligation	64
2.10. Construction of DNA libraries	65
2.10.1. Gel extraction	65
2.10.2. Rolling Circle Amplification	65
2.10.3. Digestion by restriction enzyme and cloning and sequence	65
2.11. Development of assay for gene expression analysis using quantitative real-time polymerase chain reaction (real-time-qPCR)	66
2.11.1 Generation of standard curve from plasmids of DNA clone	66
2.11.2. Components of Real Time PCR reactions	66
2.12. Rapid amplification of 5' and 3' cDNA ends (RLM-RACE)	67
2.13. Protein expression of Ro TTV transcripts	71
2.13.1. Primer design of Ro TTV transcripts amplification	71
2.13.2. Transfection of transcripts contained pEGFP-C1 and pcDNA TM 3.1/ myc-His(-) A MCS	73
2.13.3. Harvesting for western blot	74
2.13.4. Staining of Transfected Cells	74
2.13.5. Apoptosis staining by FLICA apoptosis detection kit	75
2.13.6. FACS Analysis	75

2.13.7. Confocal Microscopy	76
2.14. Western blot	76
2.14.1. Protein Electrophoresis	76
2.14.2. Transfer of Protein to Nitrocellulose Membrane	78
2.14.3. Immunological Detection of Protein Blot	78
2.15. <i>In vitro</i> experiment	79
2.15.1. Maintenance of cell line cell culture	79
2.15.1.1. Baby hamster kidney (BHK-21)	79
2.15.1.2. Mouse embryo fibroblast (MEF)	79
2.15.1.3. Passage	80
2.15.2. Primary cell culture	80
2.15.2.1. Bone marrow derived macrophage	80
2.15.2.2. Bone marrow derived stem cell	81
2.15.3. Transfection	82
2.15.4. Primers for construction of plasmid for the transfection	83
2.15.4.1. Single copy of RoTTV complete circular genome	83
2.15.4.2. Construction of GFP/RoTTV1	84
2.15.4.3. Myc tagged concatemerised RoTTV	85
2.15.4.4. RoTTV1 splicing site primer	85
2.16. <i>In vivo</i> transfection	86
 Chapter III. CHARACTERISATION OF RODENT TORQUE TENO VIRUS IN WILD RODENT POPULATIONS	 87
3.1. Background to the study	88
3.2. Infectious prevalence of RoTTV in wild rodents using pan-TTV primers	90
3.3. Sequence results of screening PCR amplicons	94
3.4. Amplification of RoTTV full-length genomes	94
3.5. Complete sequence and phylogenetic analysis of RoTTV	98
3.6. Genomic structure and coding capacity	99
3.7. Design of species specific primer sets for screening RoTTV genomic prevalence in wild rodent	105
3.8. Discussion	106

3.8.1. Distribution of RoTTV in wild rodent population	106
3.8.2. Co-evolution of TTV and host species.....	107
3.8.3. Genetic diversity of RoTTV	108
3.8.4. Genomic structure of RoTTV	110
3.8.5. Classification of RoTTV	111
Appendix 1. Rat TTV infection in lab rats	113
A.1. Background to this study.....	113
A.2. Samples	113
A.3. Screening PCR by using pan-TTV primer	113
A.4. Full-length PCR to amplify rat TTV	113
A.5. Genomic structure and coding capacity	114
A.6. Discussion	114
 Chapter IV. TRANSCRIPTS IDENTIFICATION AND PROTEIN	
EXPRESSION OF RODENT TORQUE TENO VIRUS	116
4.1. Background to the study.....	117
4.2. TTV RNA detection in systemic organ of RoTTV infected wild rodent.....	118
4.3. RACE (Rapid amplification of cDNA End) and transcript of RoTTV	122
4.4. Characterisation of the spliced TTV mRNAs and genomic organization.....	129
4.5. Predicted protein and subcellular localisation of RoTTV Fusion Protein	131
4.5.1. Prediction of RoTTV1 and RoTTV2 viral proteins	131
4.5.2. Subcellular localisation of RoTTV1 fusion protein	133
4.6. Apoptosis inducing capacity of RoTTV protein	140
4.6.1. Confocal microscopy of apoptosis stained cells	140
4.6.2. FACS analysis of apoptosis stained cells/ confocal microscopy and cell counting	140
4.7. Confirmation of viral protein expression.....	143
4.8. Discussion.....	144
4.8.1. RoTTV1 RNA distribution.....	144
4.8.2. Transcriptional profile and species specific structure of RoTTV transcript	145
4.8.3. Common splicing site of RoTTV1 and RoTTV2.....	147
4.8.4. ORF proteins of RoTTV1, predicted function, and protein localisation.....	147

Chapter V. TTV REPLICATION <i>IN VITRO</i>.....	154
5.1. Background to the study.....	155
5.2. Reagents for <i>in vitro</i> infection/transfection.....	156
5.2.1. Liver homogenate.....	156
5.2.2. Faecal supernatant.....	156
5.2.3. Single copy of RoTTV complete circular genome	156
5.2.4. Construction of GFP/RoTTV1.....	158
5.2.5. Tandem-dimerised RoTTV complete genome	160
5.2.6. Myc tagged concatemerised RoTTV	161
5.3. Bone marrow derived macrophage/Wood mouse bone marrow derived stem cell	160
5.4. Type I IFN KO mouse embryo fibroblast (MEF).....	163
5.5. Baby Hamster Kidney cell (BHK-21).....	166
5.6. Comparative study of old and young passage of BHK-21 cells	169
5.7. Virus propagation	170
5.8. Discussion.....	172
 Chapter VI. <i>IN VIVO</i> EXPERIMENT OF TTV INFECTION IN WOOD MICE AND TYPE I IFN RECEPTOR KO MICE.....	 178
6.1. Background to the study.....	179
6.2. TTV infection using concatemerised genome in wood mice/Type I IFN KO mice.	179
6.3. TTV infection using cell culture supernatant in wood mouse.....	185
6.4. Discussion.....	186
 Chapter VII. Conclusion.....	 192
 References	 197

List of Figures

Figure 1.4.1.1. Genomic organization of TTV in the prototype	21
Figure 1.4.2.1. Summary of TTV genotype 1 transcripts.	24
Figure 3.2.1. Restricted digestion of mitochondrial DNA PCR products.	91
Figure 3.3.1. Sequence of pan-TTV PCR products in wild rodents.....	92
Figure 3.2.2. Screening PCR by using pan-TTV primers	93
Figure 3.4.1. Full-length PCR of RoTTV.....	96
Figure 3.5.1. Representatives of RoTTV and other anellovirus phylogenetic tree based on partial ORF1aa sequence.	102
Figure 3.6.1 . RoTTV genomic map.	104
Figure A4.1.1. Full-length PCR of rat TTV	115
Figure A.5.1. Rat TTV genomic structure.....	115
Figure 4.2.1. RT-PCR by using RoTTV1 specific primer.....	120
Figure 4.2.2. RT-PCR by using β -actin primer	121
Figure 4.3.1. Genomic structure of RoTTV1 and RoTTV2 clones used in this study	122
Figure 4.3.2. cDNA analysis of RoTTV1 (clone; AS_HL1_Li_1)	125
Figure 4.3.3. The PCR amplified products of RoTTV mRNA in various lengths	126
Figure 4.3.4. cDNA analysis of RoTTV2 (clone AS_WM1_Se_4).	128
Figure 4.5.1.1. Arginine-rich N-terminus in ORF1 of RoTTV1 (AS_HL1_Li_1) and RoTTV2 (clone AS_WM1_Se_4)	132
Figure 4.5.1.2. The entire amino acid sequence of RoTTV1 ORF2-3 (Clone HLWM)	132
Figure 4.5.2.1.A. GFP/ORFs fusion proteins were expressed in BHK-21 cells and visualized 24 h post transfection	135
Figure 4.5.2.1.B. GFP/ORFs fusion proteins were expressed in BHK-21 cells and visualized 24 h post transfection	136
Figure 4.5.2.2.A. ORF/c-myc fusion proteins were expressed in BHK-21 cells and visualized 24 h post transfection	137
Figure 4.5.2.2.B. ORF/c-myc fusion proteins were expressed in BHK-21 cells	138
Figure 4.5.2.3. ORF1-4 and GFP fusion protein was expressed in BHK-21 cells 48 h post transfection.....	139
Figure. 4.6.1. FLICA staining in GFP and RoTTV1 ORF fusion protein.....	141
Figure. 4.6.2. Naturally apoptotic rate and fusion protein effects.....	142

Figure 4.7.1.1. Detection of recombinant RoTTV1 proteins by western blot analysis with anti GFP antibody	143
Figure 5.2.3.1. Construction of single copy of RoTTV genome.....	157
Figure 5.2.4.1. GFP/TTV construction	159
Figure 5.2.5.1. Concatemerised RoTTV genome clone construction	160
Figure 5.3.1. Bone marrow derived stem cell culture (Day 3).....	162
Figure 5.4.1. RoTTV1 and RoTTV2 transcripts were observed at 48 hours post-transfection by RT-PCR using RoTTV1 and RoTTV2 species specific primer	165
Figure 5.4.2. Experimental protocol of RoTTV1 virus replication study	165
Figure 5.4.3. RoTTV1 viral DNA copy number in the supernatant culture fluid of the P5 transfected Type I IFN KO MEF	166
Figure 5.5.1. RoTTV1 and RoTTV2 transcripts were observed at 48 hours post-transfection by RT-PCR of the concatemerised DNA transfected BHK-21 cells	168
Figure 5.5.2. RoTTV1 viral DNA titer in the supernatant culture fluid of P5 BHK-21 cells.....	168
Figure 5.6.1. RoTTV1 viral DNA copy number in the culture supernatant of RoTTV1 transfected old and young passage of BHK-21	169
Figure 5.7.1. Experimental design of RoTTV1 propagation	170
Figure 5.7.2. RT-PCR of RoTTV1 transcripts detecting the viral propagation.....	171
Figure 6.2.1. Screening of <i>in vivo</i> transfected wood mice by PCR using RoTTV1 species specific primer	180
Figure 6.2.2. Ro TTV viral DNA copy number and distribution in systemic organ	182
Figure 6.2.3. Screening PCR of Type I IFN receptor KO mice inoculated RoTTV1 concatemerised DNA	184

List of Tables

Table 2.5.1. Primer pair which were used for TTV screening and amplification of complete genome	59
Table 2.11.2. The primer pairs and probes used for the real-time PCR	67
Table 2.12.1. GeneRacer primer and race gene specific primer to detect the transcript of RoTTV1	69
Table 2.12.2. Race gene specific primer to detect the transcript of RoTTV2	70
Table 2.13.1. The primers which were used to construct the transcripts containing pEGFP-C1 and pcDNA TM 3.1/ myc-His(-) A MCS	72
Table 2.15.4.1. The primers containing the restriction site on Ro TTV genome	83
Table 2.15.4.2. The primers with the restriction sites for GFP/TTV construction.....	84
Table 2.15.4.3. Myc tagged RoTTV1 complete genome primer.....	85
Table 2.15.4.4.. RoTTV1 splicing site primer.....	85
Table 3.2.1. Host species which was investigated in this study and number of samples in each species.....	91
Table 3.3.1. Genetic distribution in each wild rodent species.....	94
Table 3.4.1. Genomes cloned in this study.....	97
Table 3.5.1. RoTTV1 genomic size and structure are shown in the table.....	100
Table 3.5.2. RoTTV2 genomic size and structure are shown in the table.....	101
Table 3.5.3. Group amino acid pairwise-distance comparisons among 17 RoTTV1, 14 RoTTV2, 129 TTV, 14 TTMV, and 21 TTMDV using partial ORF1 sequences	103
Table 3.6.1. RoTTV genomic map.....	104
Table 3.7.1. Rescreening results of RoTTV using species specific primer.....	105
Table 4.2.1. RNA distribution of RoTTV1	120
Table 4.6.2. The number of FLICA positive and GFP expressing BHK-21 cells counted to identify RoTTV1 protein apoptosis inducing capacity	142
Table 6.2.1. Initial experiment of RoTTV1.....	180
Table 6.2.2. Second experiment of RoTTV1 <i>in vivo</i> transfection.....	183

Abbreviations

Acquired immunodeficiency syndrome (AIDS)
Alanine aminotransferase (ALT)
Baby hamster kidney (BHK)
Chicken anemia virus (CAV)
Cluster of differentiation (CD)
Complementary DNA (cDNA)
Cytomegalovirus (CMV)
Deoxy nucleotide triphosphate (dNTP)
Deoxyribonucleic acid (DNA)
Dulbecco's modified Eagles's medium (DMEM)
Double stranded (ds)
Dithiothreitol (DTT)
Electron microscopy (EM)
Eosinophil Cationic Protein (ECP)
Escherichia Coli (*E. Coli*)
Exhaled Nitric Oxide (eNO)
Fluorescent Labeled Inhibitor of Caspases (FLICA)
Force vital capacity (FVC)
Force expiratory volume (FEV)
Green Fluorescent Protein (GFP)
Hepatitis-associated aplastic anemia (HAAA)
Hepatocellular carcinoma (HCC)
Hepatitis B virus infection (HBV)
Hepatitis C virus infection (HCV)
Human Immunodeficiency Virus (HIV)
Human T-cell Lymphotropic virus (HTLV-1)
Human papillomavirus (HPV)
Hypervariable regions (HVRs)
HTLV I-related retrovirus element (HRES-1/ p28)
Isoamyl alcohol iaa (IAA)
Idiopathic inflammatory myopathy (IMM)

Idiopathic pulmonary fibrosis (IPF)

Immunoglobulin (Ig)

Isopropyl β -D-1-thiogalactopyranoside (IPTG)

Messenger ribonucleic acid (mRNA)

Mouse embryo fibroblast (MEF)

Murine gammaherpesvirus-68 (MHV-68)

Nonstructural 5A (NS5A)

Phytohaemagglutinin (PHA)

polycation polyethylenimine (PEI)

Polymerase chain reaction (PCR)

Psoriatic arthritis (PsA)

Peripheral blood mononuclear cell (PBMC)

Post-weaning multisystemic wasting syndrome (PMWS)

Porcine circovirus associated disease (PCVDs)

Porcine dermatitis and nephropathy syndrome (PDNS)

Porcine respiratory disease complex (PRDC)

Porcine TTV (TTSuV)

Torque teno virus (TTV)

Representational Difference Analysis (RDA)

Rolling circle amplification (RCA)

Real-time quantitative PCR (real-time-qPCR)

Reverse transcriptase (RT)

Rodent TTV (RoTTV)

Rapid amplification of 5' and 3' cDNA ends (RLM-RACE)

Restriction Fragment Length Polymorphism (RFLP)

Revolutions-per-Minute Indicator (RPMI)

Ribonucleic acid (RNA)

Rolling circle replication (RCR)

Single-stranded circular DNA (ssDNA)

Single stranded (ss)

Small anelloviruses (SAV)

DNase-sequence independent single primer amplification (SISPA)

Systemic lupus erythematosus (SLE)

Open reading frames (ORFs)

Thermus Aquaticus (Taq)

T helper lymphocytes (Th)

Torque teno midivirus (TTMDV)

TTV-derived apoptosis inducing protein (TAIP)

TTV-like mini virus (TTMV)

Untranslated region (UTR)

Viral protein (VP)

Volume/volume (v/v)

Weight/volume (w/v)

5-Bromo-4-Chloro-3-Indolyl- β -D-Galactoside (X-gal)

CHAPTER I. INTRODUCTION

In 1997, Torque teno virus (TTV) was discovered from the serum of a patient with acute post-transfusion hepatitis of non A-G etiology and classified as genus *Anellovirus*. Anelloviruses are widely distributed in human and animal population and has extensive genetic diversity. Many aspects of TTV infection are still unknown, especially pathogenesis. No study has found TTVs related disease and TTV is an orphan virus.

1.1. *Torque teno virus* (TTV) and related *Anelloviruses*

1.2. Genetic diversity

1.3. Epidemiology/Prevalence

1.4. TTV structure and cording

1.5. Replication and Pathogenesis

1.6. Immunology of TTV infection

1.7. Model of TTV infection

1.8. Aims of project

1.1. *Torque teno virus (TTV) and related *Anelloviruses**

Torque teno virus (TTV) has been classified into a new viral family, *Anelloviridae*, and it was found in a non-A, B, C, D, or E post-transfusion hepatitis patient (Nishizawa *et al.*, 1997). The TTV genome is encased in a capsid and consists of a single-stranded circular DNA (ssDNA) ranging in size from 3.5 to 3.9 kb while the virion has been estimated to be 30-32nm by electron microscopy (Mushahwar *et al.*, 1999; Itoh *et al.*, 2000). TTV is non-envelop virus (Mushahwar *et al.*, 1999; Okamoto *et al.*, 1998). PCR analysis of human serum or plasma showed that TTV is ubiquitous, with a ~90% DNA prevalence among healthy adults (Kakkola *et al.* 2002; reviewed in Hino and Miyata, 2007). Some time after discovery of TTV, related viruses which have similar genomes were discovered and named Torque teno midi virus (TTMDV, 3.2kb) and Torque teno mini virus (TTMV, 2.8-2.9kb). Currently, TTV infection has not been linked to specific disease (reviewed in Hino and Hironori, 2007). However, we cannot exclude the possibility that TTV may damage the host, because there are reports which reveal the positive correlation between active TTV replication and severity of certain diseases including AIDS (Shibayama *et al.* 2001,) pulmonary disease (Maggi *et al.* 2003,) haematologic disorders (Miyamoto *et al.* 2000,) cancer (Zur Hausen *et al.* 2005) and autoimmune disorders (Gergely *et al.* 2005.)

1.1.1. TTV discovery

Hepatitis, inflammation of the liver, affects millions of people. Most cases are caused by hepatitis virus infection such as hepatitis A, B, C, D, and E, and donated blood is screened for Hepatitis B Virus and Hepatitis C Virus. However, there are still many hepatitis cases which cannot be ascribed to these viruses, and many researchers have tried to find the unidentified hepatitis viruses. In 1997, Nishizawa et al. applied Representational Difference Analysis (RDA) method which subtracted the host genome using virus negative sample, and amplified a new virus genome from a hepatitis patient (initials T.T.) who exhibited elevation of alanine aminotransferase (ALT) level after transfusion (Nishizawa et al. 1997). Later, this virus was named Torque teno virus (latin “torques”, necklace; “tenuis”, thin) (Biagini et al. 2004.) Further studies showed TTV from serum is sensitive to DNase I and Mung Bean Nuclease, but TTV is resistant to RNaseA and restriction enzyme. Furthermore, TTV had a density of 1.26 g/cm³ in sucrose, and it did not change after tween 80 treatment which would destroy the envelop. Moreover, when the virus genome sequence was further extended, the two extremities were connected in GC-rich stretch of about 100 nucleotides (nt) and it showed the genome was a closed circular genome. According to these studies, TTV was concluded to be a non-enveloped single stranded negative sense circular DNA virus (ss DNA) (Mushahwar *et al.*, 1999; Okamoto *et al.*, 1998). TA278, the prototype TTV isolate of the entire genome length was finally determined to be 3,853 nt with four predicted ORFs, and the unique stem loop structure in GC-rich region and rep-motif in ORF1 which would play the important role of virus replication were observed (Okamoto *et al.*, 1999b; Okamoto *et al.*, 1999c). After the discovery, it is found that TTV has high genetic variability, and more than 90% healthy individual have TTVs (reviewed in Hino and Miyata, 2007).

Its genomic organization was different from that of the other well-known single-stranded DNA

viruses such as those of the family *circovirus* (TTV was originally classified in this family). Accordingly, the new family “*Anelloviridae*,” from *anellus*, Latin for ring was created for TTV.

1.1.2. TTMV Discovery

In 2000, Takahashi et al. discovered the new small virus which had similar genomic structure to TTV and chicken anemia virus (CAV). TTV specific primers were used for the virus detection but the amplified virus was noticeably smaller than TTV. This virus was described as TTV-like mini virus (TTMV). TTMV is also negative sense single-strand closed circular DNA virus. Its particle size is estimated to be less than 30nm, and its genomic size 2.9 kb. TTMV genomic structures which have four predicted ORFs is similar to TTV, and TTMV had the GC-rich region and coding area (section 1.4.1.). TTMV was also discovered in chimpanzee and the genome size is 2785 nt. TTMV also showed the high genetic diversity.

1.1.3. TTMDV and Small anellovirus (SAV) Discovery

In 2007, Torque teno midivirus (TTMDV), a third group of the genus Anellovirus, was discovered in human serum (Ninomiya et al. 2007a) by using DNase-sequence independent single primer amplification (SISPA) and rolling circle amplification (RCA) method. TTMDV genome sizes were 3,242-3,253 nt and it is between those of TTV and TTMV. TTMDV possessed a genomic structure which contains four open reading frames (ORF1-4) with TTV characteristics, three Rep-motifs and putative stem-loop structures in GC-rich region.

Before TTMDV discovery, small anelloviruses (SAV) were detected by using SISPA method. Two new TTV like viruses were detected from the patient of acute viral infection and these two viruses named small anellovirus 1 (SAV1) and small anellovirus 2 (SAV2). SAV1 genome size is 2,249 nt with three putative ORFs. On the other hand, SAV2 had 2,635 nt genome length with

five putative ORFs (Jones *et al.*, 2005). Furthermore, by using the RCA and SISPA, SAV related viruses but with even shorter genomes, were discovered (2,002 nt and 2,454 nt) and showed 40% sequence difference from SAVs (Biagini *et al.*, 2007). SAV genomic sequence and structure is closely related to portions of TTMDV, and TTMDV was shown to be a complete virus. For this reason, SAVs were suspected to be the deleted form of TTMDV genomes or artifacts from the PCR and SAV and related viruses are not included in the current classification (Biagini *et al.*, 2009).

TTMV and TTMDV are widely distributed in human population (Ninomiya *et al.* 2007b). Currently, human TTVs are categorized in three groups; TTV in the genus *Alphatorquetenovirus*, TTMV in the genus *Betatorquetenovirus*, and TTMDV in the genus *Gammatorquetenovirus* (intergenus ORF1 nucleotide divergence greater than 56%). In this classification, 29 TTVs, 9 TTMVs, and 2 TTMDVs were classified as distinct species (previously known as genotypes) (Reviewed by Maggi and Bendinelli. 2010).

It is also suggested that these smaller viruses may be the results of the intragenomic rearrangements of complete TTV genomes (Leppik *et al.*, 2007). However, TTMV was clearly discovered as a complete virus not a satellite genome of TTVs. By the development of the new amplification method, additional TTV related virus may be discovered and further define the phylogeny of these viruses.

1.1.4. Animal TTV Discovery

TTV infection is not limited to human hosts. TTV was observed in a wide variety of species, including non-human primates (Okamoto *et al.*, 2000a; Okamoto *et al.*, 2000b; Okamoto *et al.*, 2001a; Abe *et al.*, 2000; Cong *et al.*, 2000, Romeo *et al.*, 2000; Thom *et al.*, 2003; Verschoor *et*

al., 1999), pets (Okamoto *et al.*, 2002), farm animals (McKeown *et al.*, 2004; Leary *et al.*, 1999; Brassard *et al.*, 2008) and wild animals (wild boar) (Martinez *et al.*, 2006). Entire sequences of species specific TTVs have been found in chimpanzee (Inami *et al.*, 2000), Japanese macaque (Okamoto *et al.*, 2000), cotton-top tamarin (Okamoto *et al.*, 2000), douroucouli (Okamoto *et al.*, 2000), tupaia (Okamoto *et al.*, 2001), as well as domestic animals including pig (Niel *et al.*, 2005), cat (Biagini *et al.*, 2007), and dog (Okamoto *et al.*, 2002). Although these TTV genomes shared the similar genomic structures, the genomic sizes were variable depending on the animal order. For example, TTV genome sizes of the higher animals such as a human and primates are 3.7-3.8 kb, but the genome sizes become smaller (2.8 kb) in the lower animals such as a swine and canine TTV genomes. Finally rodents which are one of the lowest mammals showed the smallest genome of TTV (2.2 kb) in current study (section 3.4). In this theory, feline TTV is the only exception because feline genome size is only 2 kb and it is the smallest genome of TTV (Okamoto *et al.*, 2002). These compete genomes are currently classified in 11 genera depending on the host species (*Alphatorquetenovirus*; Torque teno virus, *Betatorquetenovirus*; Torque teno mini virus, *Deltatorquetenovirus*; Torque teno tupaia virus, *Epsilontorquetenovirus*; Torque teno tamarin virus, *Etatorquetenovirus*; Torque teno felis virus, *Gammatorquetenovirus*; Torque teno midi virus, *Iotatorquetenovirus* and *Kappatorquetenovirus*; Torque teno sus virus, *Lambdatorquetenovirus*; Torque teno zalophus virus; *Thetatorquetenovirus*; Torque teno canis virus; *Zetatorquetenovirus*; Torque teno douroucouli virus (<http://www.ICTVdb.org/>). TTMV was also discovered in chimpanzee and is related to human TTMV, and the cross-species infection occurred between human and chimpanzee. This indicates that TTV may be zoonotic in origin (Okamoto *et al.*, 2000; Thom *et al.*, 2003).

Human TTVs and animal TTV share many characteristic including the genomic structure, wide-spread infection, and high genetic diversity. In principle, animal TTV is host species specific and

animal TTVs mentioned above, were observed only in the specific host. Human TTV is also known to infect only a human and it is very difficult to have TTV infection into other animals like mice (Isaeva *et al.*, 2002). Animal TTV genetic diversity was also found. Although the animal TTVs have not been as well studied as human TTVs, two to three genotypes were discovered in each animal. Chimpanzee has TTV and TTMV similar to human anellovirus family (Okamoto *et al.*, 2000). The genomic structures and predicted transcriptional profiles of animal TTVs were similar to those of human TTV and it is thought that co-evolution of TTVs with the hosts may be a key factor for the development of species specificity and genetic diversity (Fig. 1.3.2.). Co-infection with different sequences and genotypes of anellovirus in the same individual is common in human and animal TTV infection (Maggie and Bendinelli., 2010).

1.1.4.1. TTV Infection in Nonhuman Primates

In the chimpanzee, TTV infection is extremely common and 98% (102/104) infectious prevalence was observed in chimpanzee sera (Okamoto *et al.*, 2000; Inami *et al.*, 2000). Two chimpanzee's TTVs consisting of 3,690nt and 3,899nt were isolated and they showed 43% sequence difference from each other (Okamoto *et al.*, 2000; Inami *et al.*, 2000). Unlike most anelloviruses, Chimpanzee TTV shows interspecies infection (Mushahwar *et al.*, 1999). Due to the faecal-oral transmission route, TTV is predicted to contaminate and spread easily. Interestingly, human TTV genotype 1a was detected in one chimpanzee in Africa and the cross-species infection was also confirmed by Mushahwar *et al.*, (1999) and Tawara *et al.*, (2000). These chimpanzees had previously been used for the transmission research of human hepatitis virus and the tested 11/53 chimpanzees had an anti-human TTV genotype 1a antibody (Tusda *et al.*, 1999). Because of the discovery of chimpanzee TTMV, it is predicted that chimpanzee would have TTMDV and the dual or triple infection could occur similar to human TTV infection (Okamoto *et al.*, 2000; Ninomiya *et al.*, 2008). Thus, human and chimpanzee are considered to

share the same or similar TTVs, and chimpanzee infectious experiment has been performed by using the sera from hepatitis patients and hepatic disease developed in the chimpanzee liver (Tawara et al. 2000) (section 1.5.2.1.). In conclusion, chimpanzee TTV infection may give us the hints of understanding the origin, nature, and transmission route of human anelloviruses.

1.1.4.2. TTV infection in farm animal

TTV infection in the farm animal was reported by Leary *et al.*, (1999). In the report, TTV infection in farm animals was observed including 19% of chicken, 20% of pigs, 25% of cows and 30% of sheep by using PCR. Porcine anellovirus is well-studied as an animal model for human TTV infection (section 1.7.1.). According to the result, the cross-species infection between human and farm animal, was also suggested and it is possible that animals are the reservoir of TTVs. This story is also supported by the report showing human TTVs infection in the camels in the United Arab Emirates (AI-Moslih *et al.*, 2007). However, other studies have not supported the results (Brassard *et al.*, 2008; Okamoto *et al.*, 2002; Thom *et al.*, 2003). These cross-species infections are one of the most important topics because this result would influence the epidemiology of TTV infection in human.

1.2. Genetic diversity

After the discovery of TTV, the extremely high genetic heterogeneity was shown in the early stages of TTV research, both at the nucleotide level and at the amino acid level with 47-70% divergence among TTVs isolates (Biagini *et al.*, 1999; Luo *et al.*, 2002). The untranslated region (UTR) is relatively well-conserved and contains several motifs with greater than 90% identity (Erker *et al.*, 1999; Suzuki *et al.* 2004). Conversely, the translated regions have extensive genomic variations (Tanaka *et al.*, 2000; Biagini *et al.* 2004) in contrast to many other viruses. Until now, 5 large genogroups were found in human TTV (Biagini *et al.*, 2004). The divergence

is extremely high in the middle of ORF1, and in the case of genotype 1, there are three hypervariable regions (HVRs) (HVR1, HVR2, and HVR3). These areas contain multiple codon insertion or deletions and they make up approximately 70% of the divergence at the amino acid level (Erker *et al.*, 1999; Hijikata *et al.*, 1999; Nishizawa *et al.*, 1999; Takahashi *et al.*, 1998).

TTMV is also extremely divergent and the first three isolated TTMV had 42% nucleotide sequence difference and 67% amino acid sequence difference from each other (Takahashi *et al.*, 2000). The reasons for TTMV genetic divergence are still unknown but the recombination sites and patterns are similar to those of TTV (Biagini *et al.*, 2001). In addition, TTMDV is highly variable with divergence of up to 33% over the entire nucleotide sequence and 61% amino acid level of ORF1 (Ninomiya *et al.*, 2007).

The reasons for TTV high genetic variability are still unknown but several hypotheses have been suggested. One of the reasons would be high TTV mutation rate. Recently, virus evolutionary dynamics of ssDNA virus is well-studied, because ssDNA evolutionary rate is approaching that of RNA viruses. Indeed, ssDNA viruses including TTV show the relatively high genetic diversity (Lopez-Bueno *et al.*, 2006; Isnard *et al.*, 1998). Although ssDNA viruses (especially those with genomes smaller than ~13kb) show high mutation rate, ssDNA viruses were originally thought to have low mutation rates because ssDNA viruses depend on the host DNA polymerase for replication (Flint *et al.*, 2004). Host DNA polymerase has strong proofreading capacity unlike the reverse transcriptase coded and used by RNA viruses (Duffy *et al.*, 2008). However, ssDNA showed high substitution rate and the mutation rate is also intermediate between RNA and DNA virus according to the canine parvovirus (CPV-2) (Shackelton *et al.*, 2005), human parvovirus B19 (Shackelton *et al.*, 2006), anellovirus SEN-V (Umemura *et al.*, 2002), and plant geminivirus (Duffy *et al.*, 2008) research. It is also suggested that this high mutation rate is related to the

single stranded character of the genome and/or the coding capacity for the proteins involved in replication (Shackelton *et al.*, 2006). TTV would be expected to have a similar mechanism to make a high mutation rate.

According to the studies of virus evolution, TTV has many features contributory to the high mutation rates. It has been suggested that several factors decide the mutation and substitution rates. Firstly, TTV is ssDNA virus. ssDNA viruses have higher mutation rate compared to dsDNA viruses (Duffy, 2008). Secondly, TTV is a small (2.0-3.8kb) virus and smaller viruses like TTV tend to replicate faster which increase the chance for the virus mutation (Duffy, 2008). Thirdly, TTV is thought to use the direct infection not to use vector (Lin *et al.*, 2000). Therefore, TTV has to infect a new host by themselves and it may be required to mutate to adjust the receptor or avoid the host defence system of a new host. Finally viruses which use the horizontal infection also have to set up the robust infection as soon as possible in new host species, thus these viruses mutate frequently, and their replication speed is faster than the viruses which infect vertically (Lin *et al.*, 2000). Viruses infecting vertically sometimes become latent in the host genome and these viruses passed fewer generations compared to horizontal transmission viruses and show low mutation chances (Duffy, 2008).

The amino acid changes of TTV usually occur in the HVR of ORF1 and TTV is thought to infect the individuals as quasispecies. By the mutation of HVRs, TTV could avoid the host immune responses and establish persistent infection (Jelcic *et al.*, 2004). In TTV evolution, it was also suggested that a large number of viral particles would be produced but only a small number of these are competent and/or co-infection of different incompetent isolates made up the competent virus (Khudyakov *et al.*, 2000; Luo *et al.*, 2002). This replication system is also observed in other virus species. In the hepatitis B virus infection (HBV), HBV infected cells produce different

forms of virus related particles and only some particles can be infectious or make further virus particles (Hakami *et al.*, 2013). Furthermore, Human Immunodeficiency Virus (HIV) produces a large number of virions after infection but not all virions are competent (Pierson *et al.*, 2000).

Virus recombination is considered as a common mechanism to create new variants of viruses and, in the case of TTV, it is also suggested to occur frequently (Manni *et al.*, 2002; Worobey *et al.*, 2000). *Taq* polymerase has been shown to produce the recombinant molecules similar to the products of rolling circle DNA amplification of DNA virus. Actually, many incomplete virus genomes (shorter genome or nucleotide changes) were cloned from PCR amplicons and also TTV recombination might be a PCR artifact (Jelcic *et al.*, 2004; Zaphiropoulos, P. G., 1998), but this idea has been challenged (Worobey *et al.*, 2000). Recently, Leppik *et al.* found several fragments of transcripts of TTV in a Hodgkin's lymphoma, and it may indicate that intragenomic rearrangement has occurred in the host cells. It is also believed that the genetic evolution and genomic rearrangement are related to TTV heterogeneity and pathogenicity (Hino and Miyata. 2007; Leppik *et al.* 2007). TTV genetic variability makes TTV research difficult. Much effort has been spent to design primers able to amplify the variable TTVs and related viruses genome in TTV research (section 1.3.1) and the different TTVs may have different nature. For example, as high human TTV genotype 1 infectious prevalence in the patients, human TTV genotype 1 is considered to be more pathogenic than other TTVs (section 1.5.2.1.), and different TTVs may have different replication sites and pathogenesis (section 1.5.2.). However, it could also be indicated that human TTV genotype 1 may be more commonly infected in healthy individuals and patients than other genotypes, and genotype 1 may be more detectable by PCR than other genotypes in the patients not to indicate that human TTV 1 is more pathogenic.

Despite this wide variation in sequence, the TTV UTR sequence is well conserved (Hino and Miyata. 2007; Suzuki *et al.*, 2004; Kamada *et al.*, 2004). Replicating TTVs reproduce the large number of virions, for example; 1.0×10^4 - 2.1×10^6 copies/ml in hepatic carcinoma patient (Tokita *et al.*, 2002). Due to the high replication, hypermutation or recombination could occur and it is speculated to contribute to the viral evolution (Duffy *et al.*, 2008). However, these mutations have a certain level of limitation because the mutation may cause a functional deficiency for the replication in the host cells. Thus, TTV might need to conserve some sequences or structures and these areas may have an important role in TTV replication. Furthermore, virus should not turn to be excessive virulent virus. Successful viruses would cohabit with host for replicating efficiently. Therefore, TTV infectious prevalence is extremely high, but the fataling rate is very low.

It is suggested that this TTV genetic diversity is thought to come from the host-virus co-evolution in different host species and it would take millions of years (Ball *et al.*, 1999; Biagini *et al.*, 1999; Hijikata *et al.*, 1999; Prescottte *et al.*, 1999; Gallian *et al.*, 2000). This idea would be contradictory to the other researcher's ideas mentioned above, and some researchers believe that TTV evolution occurs very slowly (Khudyakov *et al.*, 2000). One reason is TTV titre is usually very low in the host and this indicates that TTV replication speed is slow (Okamoto *et al.*, 1999). Low replication rate would be predicted to lead to a low mutation rate because the virus has passed fewer generations and therefore, mutation chances. Also, it has been suggested that TTV primary genomic structure is highly stable and one study reveals that the TTV genome did not change over a 31 month observation time (Biagini *et al.*, 1999; Khudyakov *et al.*, 2000). This contradicts the hypothesis proposed by a huge number of TTV heterogeneity papers (Worobey *et al.*, 2000). Thus, it is concluded that long history of human and animal infection of TTV makes the TTV heterogeneity (Khudyakov *et al.*, 2000). In addition, it was suggested that multiple

infection of different TTV genotypes enhances mutation frequency (Manni *et al.*, 2002). The mechanism of TTV divergence is still unknown but it is revealed that human TTV infection happens not only with one TTV species but with a large family of TTVs. Some viruses show multiple infections in the host cells and produce the different genotypes of virus to avoid the host immune system. For example, the multiple genomes of HIV-1 infect in the same cells, produce different genetical proviruses and HIV-1 produces the drug resistance virus via recombination (Dixit and Perelson, 2004; Christian *et al.*, 2012). TTV multiple infection would induce the virus recombination to produce further genotypes.

1.3. Epidemiology/Transmission

1.3.1. Epidemiology

After first identification of TTV, epidemiological research was performed to understand TTV infectious prevalence and TTV infection was shown in healthy individuals and various patients indicating the possible disease associations (section 1.5.). However, the result was not reliable and speculated to be low infectious prevalence compared to the actual infectious rate because the wide sequence variation was not known at the time (section 1.2.). The PCR conditions, especially primer design, has a large impact on the PCR results and some primers tend to detect fewer genotypes than others (Ali *et al.*, 2002; Irving *et al.*, 1999; Leary *et al.*, 1999; Lopez-Alcorocho *et al.*, 2000; Niel *et al.*, 1999; Nishiguchi *et al.*, 2000; Okamoto *et al.*, 1999b; Pollicino *et al.*, 2003; Ukita *et al.*, 2000). For the current method, based on the knowledge of the genomic variation, various primer combinations have been used to detect TTVs quantitatively and qualitatively (Biagini *et al.*, 2001a; Devalle *et al.*, 2004; Hu *et al.*, 2005; Maggi *et al.*, 2003b; Moen *et al.*, 2002b). However, it was still difficult to amplify the animal TTVs; for example, N22 primers which were designed based on ORF1 used for TTV screening in early TTV studies, could not amplify animal TTV from any nonhuman primate serum (Okamoto *et al.*, 2000). After the

study, taking advantage of the highly conserved UTR sequence, broader spectrum primers to detect various TTVs of animals were designed and many animal TTV species were discovered (Okamoto *et al.*, 2001; Okamoto *et al.*, 2002). Use of these primers extended the scope of TTV research and has played a key role in new TTV discovery, the understanding of TTV viral character, and the evolutionary relationship among TTVs.

PCR has remained the standard for TTV detection but the result is influenced by the primer design. Sequence independent methods including Rolling circle amplification (RCA), have also been developed recently. This method has several advantages for amplification of circular viruses such as strand displacement activity, proof reading activity, generation of long synthesis products, and a relatively straightforward technique (30°C overnight) (Reimer *et al.*, 2009). In RCA, a random hexamer primer attaches to multiple sites of template circular DNA and from the sites, DNA is isothermally extended by phi 29 DNA polymerase (Garmendia *et al.*, 1992; Esteban *et al.*, 1993). By using this method, new circovirus (Johne *et al.*, 2006), geminivirus (Haible *et al.*, 2006), and TTVs were detected (Niel *et al.*, 2005; Macera *et al.*, 2011). Furthermore, the combination of RCA and a new method, sequence independent single primer amplification (SISPA), were applied to detect TTVs in human plasma and cat saliva (Biagini *et al.*, 2007). This method requires target DNA and asymmetric linkers or primers which contain restriction endonuclease sites (Reyes and Kim 1991; Allander *et al.*, 2001). First the target DNA is digested by Mbo-I. Linkers which have the same restriction sites (Mbo-I) connect to the digested DNA and PCR is performed by using the linker primer. It has been suggested that this new technique, RCA and SISPA, would be useful tool for detecting more divergent viruses.

By using PCR and new methods, TTV infectious prevalence is considered to be extremely high in human and animals. In terms of prevalence, it is indicated that TTV is one of the most

successful viruses in a number of species. TTV is found world-wide in the general population (Abe *et al.*, 1999; Okamoto *et al.*, 1999; Prescott *et al.*, 1998; Viazov *et al.*, 1998) and their level of infection increases with age regardless of gender, economical condition, and geological location (Hsieh *et al.*, 1999; Saback *et al.*, 1999; Umemura *et al.*, 2001; Zhong *et al.*, 2001). Although, Infectious prevalence varied depending on TTV detection methods and primer difference for the PCR (Leary *et al.*, 1999), it has been demonstrated that TTV occurs all over the world including Brazil (Devalle *et al.*, 2004; Niel *et al.*, 1999), Turkey (Erensoy *et al.*, 2002; Yazici *et al.*, 2002), Norway (Huang *et al.*, 2001), China (Zhong *et al.*, 2001), but geographical differences in prevalence were not observed (Huang *et al.*, 2001; Manni *et al.*, 2002; Mushahwar *et al.*, 1999; Pisani *et al.*, 1999). Currently two-thirds or more of global populations have TTVs. TTMDV and TTMV are predicted to show the same level of infection. Furthermore, multiple infection of TTV and TTV-related small virus is common (Bendinelli *et al.*, 2001; Moen *et al.*, 2002; Maggi *et al.*, 2005; Biagini *et al.*, 2006a; Biagini *et al.*, 2006b; Ninomiya *et al.*, 2008). Although the prevalence would change over the time depending on the technological development, it is considered TTV and TTV-related small virus showed the extremely high infectious prevalence.

Epidemiology of TTMV and TTMDV

TTMV is also distributed worldwide among healthy individuals (Biagini *et al.*, 2001; Niel *et al.*, 2001). TTMV DNA prevalence in the blood donors was studied to be 48-76.9% in different counties such as 62% in France (Biagini *et al.*, 2006), 67-72% in Brazil (Devalle *et al.*, 2004; Niel and Lampe, 2001), 48% in the Norway (Moen *et al.*, 2002), and 76.9% in Japanese pregnant woman (Matsubara *et al.*, 2001). Furthermore, TTMV isolates were also found in various tissues such as serum/plasma, PBMC, faeces, saliva, bone marrow, spleen, and cervical swabs with various viral loads (1.3×10^3 - 1.7×10^8) (Biagini *et al.*, 2001; Fornai *et al.*, 2001; Thom *et al.*, 2003;

Vasconcelos *et al.*, 2002).

TTMDV infectious prevalence is still required to be studied by using accurate methods, and TTMDV is also predicted to spread in general populations according to several reports studied about TTMDV infectious prevalence in plasma samples from European and Japanese blood donors (Jones *et al.*, 2005; Ninomiya *et al.*, 2008; Andreoli *et al.*, 2006; Biagini *et al.*, 2006). Studies have been performed on SAV which is suspected to be related to, or a laboratory artifact of TTMDV. SAV is distributed in systemic organ and body fluid including saliva, PBMC (Biagini *et al.*, 2006), and nasopharyngeal aspirates (Chung *et al.*, 2007). SAV is also common in the healthy individuals and it was observed in 20% of French blood donors (Biagini *et al.*, 2006) and 34.5% of Korean children (Chung *et al.*, 2007). TTMDV and SAV have not been as well studied as TTV and these infectious prevalence measurements could be changed depending on the primer design mentioned above. Accordingly, it is predicted that TTMV and SAV infect ubiquitously similar to TTV infection. In addition, most infected people have more than one genotype of TTV or/and the related smaller genome viruses (Maggi *et al.*, 2005).

1.3.2. Transmission

1.3.2.1. Vertical transmission

Anellovirus infections are generally thought to occur in the early stages of life. A report which studied TTV infection in cord blood samples and serum samples from children 1 year and older, showed anellovirus was negative in cord blood samples but within 2 years TTV infectious prevalence rose to 100% (Ninomiya *et al.*, 2008). Based on these studies, TTV transplacental transmission has only a minor role in TTV infection (Mutlu *et al.*, 2007; Ninomiya *et al.*, 2008). However, it is very difficult definitively to prove a lack of TTV because of the issue surrounding PCR sensitivity and low virus titer. When TTV sequence was compared between mother and

children, children showed TTV sequences consistent with both vertical and environmental transmission (Davidson *et al.*, 1999; Sugiyama *et al.*, 1999; Bagaglio *et al.*, 2002; Lin *et al.*, 2002; Ohto *et al.*, 2002). Therefore, mother-to-fetus transmission may explain some parts of TTV high and persistent infectious character (Morrica *et al.*, 2000).

1.3.2.2. Horizontal transmission

TTV infections are believed to be predominantly transmitted via the horizontal route. TTV and other anellovirus genomes have been found in the faeces (Biagini *et al.*, 2001; Okamoto *et al.*, 1998), saliva (Biagini *et al.*, 2001; Deng *et al.*, 2000; Gallian *et al.*, 2000; Goto *et al.*, 2000; Ishikawa *et al.*, 1999; Vasconcelos *et al.*, 2002) breast milk (Gerner *et al.*, 2000; Goto *et al.*, 2000; Schröter *et al.*, 2000), genital secretion (Reviewed by Bendinelli *et al.*, 2001), tears (Emre *et al.*, 2007), skin and hair follicles (Osiowy *et al.*, 2000), suggesting several potential sources for transmission of TTV. Several papers investigated the possible transmission routes of TTV.

Parenteral infection

TTV is frequently referred to as a transfusion transmitted disease because TTV is found in a myriad of tissues and body fluids and it can be transmitted by blood transfusion. TTV was discovered in post-transfusion hepatitis patient. For this reason, blood and blood products have been vigorously studied. TTV DNA prevalence is higher in patients who have several blood transfusions (Viazov *et al.*, 1998; Simmonds *et al.*, 1998; Forns *et al.*, 1999; Gallian *et al.*, 1999; Kanda *et al.*, 1999; Takayama *et al.*, 1999; Prati *et al.*, 1999; Maeda *et al.*, 2000; Amarante *et al.*, 2007), although some researchers have found the opposite (Schröter *et al.*, 1998; Rubinstein *et al.*, 2000). Furthermore, TTV was detected in blood products such as commercial human plasma (Pisani *et al.*, 1999; Simmonds *et al.*, 1998; Yokozaki *et al.*, 1999), first-generation recombinant factor VIII concentrates, factor IX concentrates (Azzi *et al.*, 2001; Simmonds *et al.*, 1998;

Yokozaki *et al.*, 1999; Chen *et al.*, 1999), and intramuscular immunoglobulin (Ig) preparations (Pisani *et al.*, 1999; Simmonds *et al.*, 1998). However, TTV infectious prevalence is too high in human and animal populations to be explained infecting solely using blood transfusion. Thus, the name, transfusion transmitted virus is misleading because it is likely that other infectious routes are more important for TTV infection.

Faecal-Oral transmission

Faeces contains TTV (Okamoto *et al.*, 1998; Ross *et al.*, 1999; Ukita *et al.*, 1999; Lin *et al.*, 2000) and some reports have shown that this faeces is infectious in cell culture (Maggi *et al.*, 2001) and non-human primates (Luo *et al.*, 2000) suggesting faecal-oral route as a most common transmission route. TTV exhibits high resistance of virions to environmental inactivation. By comparing to the structurally similar virus (Porcine circovirus 2 (PCV-2) and CAV), TTV should be resistant to physicochemical agent (Welch *et al.*, 2006) and TTV can survive the depth filtration procedure (Azzi *et al.*, 2006). Also, TTV is widespread in the environment and it was already investigated that TTV is detectable in river water (Hiramoto *et al.*, 2005; Verani *et al.*, 2006; Hiramoto *et al.*, 2010), sewage (Vaidya *et al.*, 2002; Hiramoto *et al.*, 2005; Hiramoto *et al.*, 2008), polluted superficial waters (Hiramoto *et al.*, 2008; Diniz-Mendes *et al.*, 2008), and drinking water (Griffin *et al.*, 2008). Furthermore, TTV and TTMV were found in 8% of Norwegian shellfish (Myrmel *et al.*, 2004) suggesting that TTV could infect using food borne transmission. Therefore, it is thought that faecal-oral route is main route of TTV infection.

Respiratory infection

Another potential route of TTV infection is through the respiratory tract as TTV replication is predicted in this site (Maggi *et al.*, 2003a; Maggi *et al.*, 2003b; Maggi *et al.*, 2004; Bando *et al.*, 2008). This idea is also supported by the studies about TTV infection in respiratory disease

patients (section 1.5.2.2.) (Bando *et al.*, 2001; Pifferi *et al.*, 2005; Pifferi *et al.*, 2006; Chung *et al.*, 2006). Recently, exhaled breath was studied for airborne TTV infection, and it showed the possibility to transmit by the breath in the same room (Chikasue *et al.*, 2012).

Sexual transmission

Sexual transmission has also been studied as another potential infectious route of TTV to spread in the adult population. TTV was detected in cervical swabs (Calcaterra *et al.*, 2001; Chen *et al.*, 2001; Fornai *et al.*, 2001) and in semen (Inami *et al.*, 2000; Martinez *et al.*, 2000) indicating that sexual transmission is possible.

1.4. TTV structure and coding

1.4.1. Genome structure

At the end of 1999, the genome structure of TTV was shown in two papers and complete genome sequences from 10 TTV isolates which have genome sizes between 3,808 nt (SANBAN isolate) and 3,853 nt (isolates TA278 and JA20) were studied (Miyata *et al.*, 1999; Erker *et al.*, 1999). Although, the described genomic sequences were variable, some parts of genome were conserved. Based on the sequence results which had well-conserved area among the sequenced isolates, TTV genome is divided into a potential coding region of ~2.6kb and an untranslated region of ~1.2kb (Fig. 1.4.1.1.) (Miyata *et al.*, 1999; Kamada *et al.*, 2004; reviewed in Beninelli *et al.*, 2001). The former region contains two major partially overlapping protein –coding genes; open reading frame 1 (ORF1) and ORF2 and it was also predicted to encode the further potential ORFs. Prototype TA278 sequence, ORF1 spans nt 589 to 2898 and ORF2 spans nt 107 to 712, but the sequence variation at the nucleotide level is quite high among TTV genotypes. A small additional ORF3 was also discovered downstream of ORF1 and is well conserved (Fig. 1.4.1.1). In addition, there are several conserved structures in the TTV genome. For example, TTV

genome contained unique stem loop structures in a GC-rich region (GC contents; 89%-90.6%) which have around 100 nucleotides (Miyata *et al.*, 1999; Hallett *et al.*, 2000; Heller *et al.*, 2001; Peng *et al.*, 2002). A poly-A sequence downstream and a TATA-box upstream of the coding region are also well-conserved (Erker *et al.*, 1999; Hallett *et al.*, 2000; Okamoto *et al.*, 2000; Heller *et al.*, 2001; Mueller *et al.*, 2008). At the nucleotide level, the untranslated region (UTR) is more conserved than the coding area, and it contained the basal promoter and enhance elements for transcriptional regulation (Suzuki *et al.*, 2004; Kamada., 2004). Therefore, PCR primers for screening TTV in human and animals are most commonly designed based on the UTR (Okamoto *et al.*, 2000; Okamoto *et al.*, 2001; Okamoto *et al.*, 2002; Thom *et al.*, 2003).

TTV was historically classified as a member of the *circovirus* family because the TTV genomic structure is similar to that of chicken anemia virus (CAV) in *circovirus* family (Kooistra *et al.* 2004). CAV is currently the only virus in the genus *Gyrovirus* of the *Circoviridae* because CAV is different from the other circoviruses in a few aspects. Circoviruses have an ambisense genomes but CAV has a unidirectional genome which is also seen in TTV. CAV also lacks the potential stem-loop structure which is common in Circoviruses. Furthermore, configurations of ORFs which have partially overlapping ORF2 and ORF1 in CAV and TTV are similar. A non-coding region with GC-rich sequence was conserved in TTVs and CAV. Also, a 36-nt region (nt 3816 to 3851) of TTV was 80.6% identical with that of CAV (Miyata *et al.*, 1999; reviewed in Hino and Miyata., 2007). However, no other parts of CAV and TTV structure were similar, and in particular TTV does not have the CAV unique region which was shown as four or five near-perfect direct repeats of 21bp (Noteborn *et al.*, 1994). Thus, CAV is currently classified into "*Circoviridae*, *Gyrovirus*" (<http://www.ictvonline.org/virusTaxonomy.asp>) but possibly could be re-classified into *Anelloviridae* due to its similarities to TTVs. Transcription profiles with spliced transcripts of CAV were similar to those of TTVs (Kamada *et al.*, 2006) and some protein

functions of TTV and CAV were closely related to each other (section 1.4.3.).

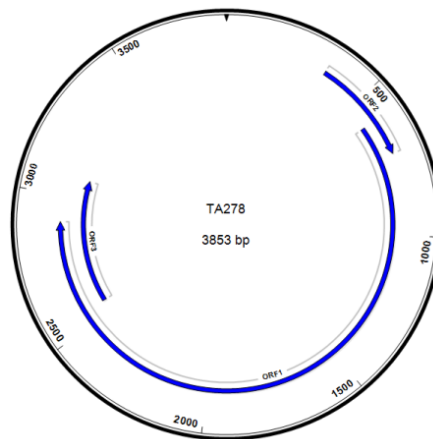


Figure 1.4.1.1. Genomic organization of TTV in the prototype TA278 (Accession number: AB017610) sequence showing single strand circular DNA of negative polarity encapsidated into the virion.

Finally, the UTR is known to function in transcriptional control because of the presence of transcription factor binding sites in the region (Miyata *et al.*, 1999). Moreover, the promoter and enhancer elements have mapped to the UTR (Kamada *et al.*, 2004; Suzuki *et al.*, 2004). The promoter/enhance activity of the different TTV genotypes was variable depending on the types of cells. This may be related to the cell tropism of TTV infection and different genotypes may have different transcriptional regulation activity (Kamada *et al.*, 2004). In addition, not all enhance motifs in the UTR were conserved. This indicates that different host cells and TTV genotypes have different transcriptional regulation system (Kamada *et al.*, 2004).

1.4.2. Transcriptional Profile

According to the complete genome analysis of original clone (TA278) (Fig. 1.4.1.1.), 2-3 predicted open reading frames (ORFs) were observed (reviewed in Hino and Miyata., 2007).

However, it does not mean that these ORFs were actually transcribed. Due to the lack of a cell culture system, TTV transcription profile has largely come from the cells transfected with human TTV complete genome. Using the strategy, transcriptional profiles from Genotype 1a transfected Huh7 and Cos-1 cells and Genotype 6 293 cells clones have been published (Kamahora *et al.*, 2000; Qiu *et al.*, 2005; Mueller *et al.*, 2008).

TTV genotype 1a complete genome was transfected in the monkey kidney cell line Cos-1 and three clone-derived mRNAs; 3.0, 1.2 and 1.0 kb in length were detected in the transfected cells (Kamahora *et al.*, 2000). In addition, the shortest mRNA of 0.6 kb in length was discovered later (Mueller *et al.*, 2008). The 5'-end and 3' end of the larger three mRNAs are identical (Qiu *et al.*, 2005). The transcripts share the same poly-A at the end of coding region and started between predicted TATA box and first methionine of ORF2. All the transcripts showed the same splicing site toward 5' end of the mRNA. The 3.0kb transcripts contained the complete ORF1 (frame 1) and ORF2 (frame 2) coding sequences. The 1.2kb mRNA had a second splicing site and ORF2 (frame 2) connected to a downstream ORF (frame 2) to create the new ORF (ORF2/2) in the same reading frame and ORF1 (frame 1) spliced and connected same ORF1 to construct shorter ORF1 (ORF1/1). The 1.0 kb mRNA also had a second splicing site but this time introduces a frame shift and that ORF2 (frame 2) connected to a downstream ORF (frame 3) to construct the new ORF, ORF3 and ORF1 (frame 1) connected an ORF in frame 2 to make ORF1/2 (Mueller *et al.*, 2008). Recently, a small 0.6 kb small mRNA which had a second splicing site was found and ORF2 (frame 2) connected to a downstream of ORF (frame 3) to create the new ORF (ORF4) (Mueller *et al.*, 2008). The transfection was also performed in 293 (human embryonic kidney cells) and L428 cells (nodular sclerosing Hodgkin's lymphoma), and the three larger of mRNAs were also detected in these cell types (Kamahara *et al.*, 2000; Okamoto *et al.*, 2000; Qiu *et al.*, 2005; Leppik *et al.*, 2007). Similar results were shown in bone marrow cells from a patient who

was infected by TTV (Okamoto *et al.*, 2000). An additional splicing site was discovered in L428 Hodgkin's lymphoma cells which were transfected with tth7 and tth8 isolates (Jelcic *et al.*, 2004). The transcription map of genotype 1 (P/1C1 clone) TTV generated by TTV transfected Huh7 cells is shown in Fig. 1.4.2.1. In all, seven possible proteins (six predicted proteins from the three large transcripts in Qiu *et al.*, 2005 and one newly described in Mueller *et al.*, 2008) are encoded by this virus.

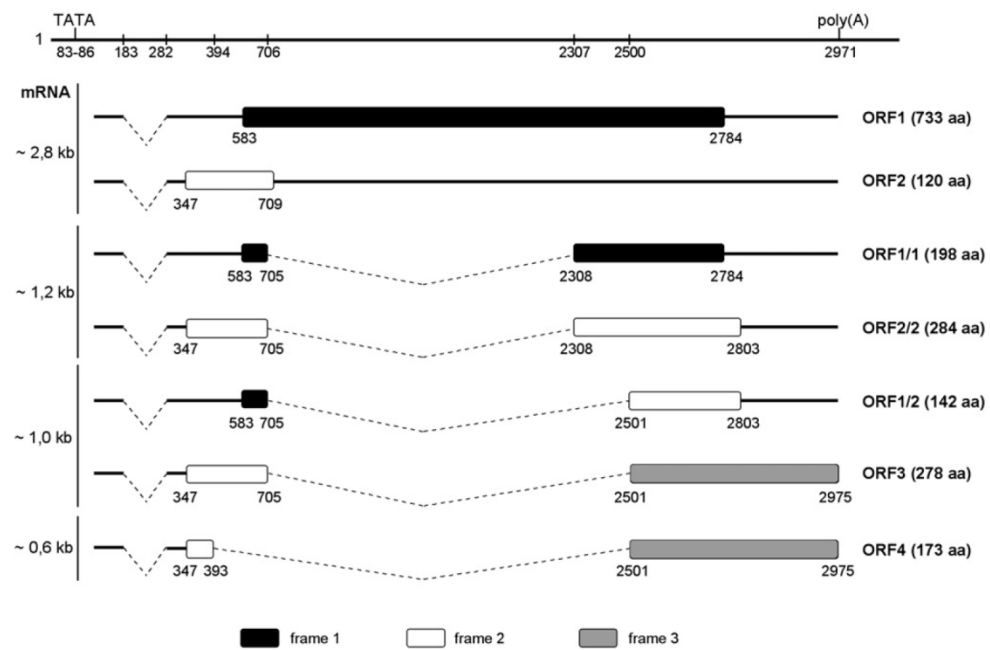


Figure 1.4.2.1. Summary of TTV genotype 1 transcripts. Genetic map of TTV is shown in the top and initiation site, all splicing site, and polyadenylation cleavage site were indicated. Seven potential ORFs expressions are diagrammed in the map below. (Reproduced from Mueller *et al.*, 2008)

1.4.3. Proteins

As documented in section 1.4.2., by variable splicing and alternative initiation up to 7 predicted proteins can be encoded by the overlapping ORFs of TTV. However, the functions of the proteins of TTV are poorly understood due to the lack of a cell culture system.

Protein localisation

The proteins from their transcripts from P/1C1 genotype1 were expressed in human 293, Huh and Hela cells, and ORF1/1 and ORF1/2 proteins were found in the nucleoli (Mueller *et al.*, 2008). ORF2 was distributed in a reticular structure of the cytoplasm (Mueller *et al.*, 2008). ORF2/2 was demonstrated in nucleoli or in the nucleoplasm. ORF2/3 and ORF4 (Fig. 1.4.2.1.) were detected in the nucleoplasm (Mueller *et al.*, 2008). However, the location of ORF1 protein was different between an older report of genotype 6 and a more recent report of P/1C1 genotype1. Genotype 6 ORF1 was located in the cytoplasm (Qiu *et al.*, 2005), but P/1C1 genotype1 ORF1 was expressed in the nucleoli (Mueller *et al.*, 2008).

ORF1: The longest ORF in all anellovirus is named ORF1. In human TTV, ORF1 is believed to contain a structural protein related to a capsid protein (Takahashi *et al.*, 1998) and the predicted length of the ORF1 encoded protein was variable around 719-770 amino acids (Erker *et al.*, 1999; Heller *et al.*, 2001., Tanaka *et al.*, 2000; Ukita *et al.*, 2000). In some isolates, premature stop codons in the middle of TTV ORF1 were observed suggesting the production of the defective TTV genomes (Erker *et al.*, 1999; Luo *et al.*, 2000; Khudyakov *et al.*, 2000; Pollicino *et al.*, 2003; Jelcic *et al.*, 2004) or full length ORF1 might not be necessary for virus replication. Furthermore, in the same genotype, nonsynonymous changes can be observed in ORF1 (Mushahwar *et al.* 1999; Ukita *et al.*, 2000). Therefore, TTV is considered to have a quasispecies nature and in this, the ORF1 change would play an important role in the evasion of the host

immune surveillance and establishment of persistent infection. This quasispecies nature is also observed in HCV and HIV-1 and the hyper variable region (HVRs) of human TTV genome which contains aa 286 to 403 (TA278, SANBAN, and TUS01), worked similar to HVRs of hepatitis C virus (HCV) (HVRs in the E2 region), HIV-1 (V3 loop) (Hijikata *et al.*, 1991; Meyerhans *et al.*, 1989; Yamaguchi and Gojobori *et al.*, 1997; McAllister *et al.*, 1998; Nishizawa *et al.*, 1999; Umemura *et al.*, 2002), or human T-cell leukemia virus-1 (the *tax* gene) (Umemura *et al.*, 2002). The TTV ORF1 HVRs are sites of high frequency nonsynonymous nucleotide changes which cause amino acid changes, some of which are removed by host immunity leaving persistent variants remaining (positive selection). On the contrary, the most conserved area in ORF1 is first 65aa which have an arginine-rich region and this area has a homology of 56.5-70% among TTVs. It has also been suggested that ORF1 protein is associated with TTV replication and contains two rolling circle replication motifs (Rep-motifs) which are conserved among the various isolates (Bendinelli *et al.* 2001; Takahashi *et al.* 1998). Viral protein (VP) 1, the capsid protein of CAV also has Rep-motifs and an arginine-rich N-terminus similar to TTV ORF1, and could function as a replication associated protein (Takahashi *et al.*, 1998; Niagro *et al.*, 1998). Interestingly, the expression of TTV ORF1 in transgenic mice caused renal epithelial cell change (Yokoyama *et al.* 2002). This indicate that TTV ORF1 could relate to renal pathogenicity.

ORF2: ORF2 is also observed in all human and animal TTV (Okamoto *et al.*, 2009) and is located upstream of ORF1 with an overlapping region (Takahashi *et al.*, 1998). This structure was also found in CAV genome structure (Takahashi *et al.*, 1998). ORF2 is assumed to code for a nonstructural protein involved in viral replication (Okamoto *et al.*, 1998. Reviewed in Bendinelli *et al.*, 2001), and in the some clones, the stop codon was observed in the middle of ORF2 and the subsequent ATG codon starts translation downstream. Accordingly, ORF2 was divided in two smaller protein coding areas, ORF2a and ORF2b (Tanaka *et al.*, 2000). ORF2a is well conserved

(more than 85-90%) compared to ORF2b (less than 70%) (Tanaka *et al.*, 2000; Ukita *et al.*, 2000). TTV ORF2 coding sequence of genotype 3(SANBAN) have a high amino acid similarity (53-55%) with CAV VP2. This TTV and CAV ORF2 similarity is also observed in Sanban, Yonban, and TTMV, and these anelloviruses have the same WX₇HX₃CXCX₅H sequence as CAV VP2 (Peng *et al.*, 2002; Peter *et al.*, 2002). The CAV VP2 has protein-tyrosine phosphatase (PTPase) activity and regulates the cellular and/or viral protein in the infection (Peter *et al.*, 2002). TTV may have similar host immune regulation system like CAV. In a recent report, TTV ORF2 protein (SANBAN isolate) was found to suppress both canonical and noncanonical NF- κ B activity. That fact indicates that the TTV ORF2 may indirectly decrease the expression of inflammatory cytokine and contribute to TTV pathogenesis (Zheng *et al.* 2007).

Other ORFs: The other TTV ORFs are created by the splicing and the nomenclature is not constant and varies among the publications. The expressed ORF2/2 and ORF3 (OR2/3) proteins of genotype 6 from 293 cells (corresponding to the ORF2-4 and ORF2-5 proteins of genotype 1 respectively; Kamahara *et al.*, 2000) were located in the nucleus (Asabe *et al.*, 2001; Qiu *et al.*, 2005) and they may be indicated to have functions in genome expression and replication. The ORF2/2 is approximately 280 amino acids (nt 347 to 705 and nt 2308 to 2803) and has a serine-rich domain (Fig. 1.4.2.1.) (Asabe *et al.*, 2001; Tanaka *et al.*, 2001; Peng *et al.*, 2002). This ORF2/2 of genotype 1a had a similar genomic sequence to hepatitis C virus(HCV) nonstructural 5A (NS5A) protein which is phosphoprotein and it is believed to be associated with various cellular proteins (Asabe *et al.*, 2001). In the COS-1 cells, TTV ORF2/2 protein could be phosphorylated and its genome produced two forms of protein with different level of phosphorylation. It has been suggested that TTV ORF2/2 has similar functions to HCV NS5A and regulates viral replication and cellular replication (Asabe *et al.*, 2001). However, the actual function of viral protein in TTV infection cycle was still unknown.

ORF4 encodes a protein with approximately 280 amino acids (nt 347 to 393 and 2501 to 2975). ORF4 is also predicted to have a function to regulate TTV transcription because it has similarity to proteins with transcription-factor activity (Kamahora *et al.*, 2000). Also, ORF4 is conserved at the amino acid level among TTVs but the function is still unknown (Okamoto *et al.*, 2000; Peng *et al.*, 2002).

The genomic organization of TTV is quite similar to that of chicken anemia virus (CAV). TTV putative protein from ORF2 is quite similar to that of the CAV VP3 protein. CAV VP3 protein induces p53 independent type of apoptosis in cancer cell line. This TTV protein named TTV-derived apoptosis inducing protein (TAIP), has been shown to induce apoptosis in hepatocellular carcinoma cells (Kooistra *et al.* 2004). However, it is still not clear whether ORF2 coded by other genotypes express this protein.

Furthermore, there are possibilities that other TTV proteins in addition to the proteins shown above exist. Recently TTV intragenomic rearrangement was suggested from TTV transfected into the Hodgkins-lymphoma cells and it makes another splicing site to create new ORFs. Thus, further variation of TTV coding capacity was predicted (Leppik *et al.*, 2007).

1.4.4. Genomic structure and predicted proteins of TTMV, TTMDV and SAV

TTMV has also long ORF1 preceded by a short ORF2 and GC-rich region, the coding areas of which were similar to those of TTV. As shown in CAV VP2 protein research, the ORF2 protein of TTMV had a function to act as a dual-specificity phosphatase (Peters *et al.*, 2002). Moreover, TTMV and CAV have an additional ORF3 (Takahashi, *et al.* 2000). This ORF3 was indicated to have a serine-rich C-terminus and it is corresponding to TTV ORF3 having function like DNA topoisomerase I. This protein is known to catalyze breakage of single stranded DNA and plays

important role of ssDNA virus replication (Peters *et al.*, 2002). The ORF1 TTMV also had an arginine-rich N-terminus and Rep-motifs similar to TTV. The untranslated region of TTMV was similar to that of CAV in that both viruses had direct repeat sequence which are absent from TTV although the repeated sequence units are different (section 1.4.1.) (Takahashi *et al.*, 2000). A chicken anemia virus (CAV) like motif in the ORF2 region (WX7HX3CXCX5H) was also observed (Takahashi *et al.*, 2000). Four sequence motifs were predicted in many TTMV isolates and conserved motifs 1 (FTL/FxTL), 2(HxH), and 3(YxxK) were identified in ORF1 but motif 4(GxxxxGK5) and the P-loop (putative ATP/GTP-binding motif), which were observed in TTV and CAV, were not found (Biagini *et al.*, 2001). These features indicated that TTMV has an intermediate structure between TTV and CAV (Biagini *et al.*, 2001; Biagini *et al.*, 2007; Okamoto *et al.*, 2000; Takahashi *et al.*, 2000).

The transcriptional profile of small anellovirus and TTMDV has not been studied yet but it is known that SAV and TTMDV genomic structures are closely related. SAVs had similar structure of TTVs and TTMV; a largest ORF (ORF1), a GC-rich region and ORF2 which had CAV like motif (Andreoli *et al.*, 2006).

1.5. Replication and Pathogenesis

1.5.1. Replication mechanism

The exact mechanism of TTV replication system is still unknown, but some researchers have predicted the TTV replication system based on other small DNA viruses. Most small DNA viruses utilize the host cell replication system for virus replication. TTV is not predicted to encode a DNA polymerase based on the genomic analysis. Viruses requiring a host polymerase either infect highly replicating cells or induce their host cells into S-phase (Kakkola *et al.*, 2007). In aphidicolin (an inhibitor of eukaryotic nuclear replication) treated 293 T cells, DNA

replication of TTV was totally blocked by the drug indicating that TTV depends on cellular replication machinery (Kakkola *et al.*, 2007). Also the virus replication process is predicted to depend on the host cell topoisomerase or helicase to gain the local binding and/or unwinding function in the virus replication. This replication method was observed in rolling circle DNA replication mechanism of bacteriophage 8X-174 and other viruses (Niagro *et al.*, 1998; Iiyama *et al.*, 1992). It is predicted that CAV and TTV would apply the similar replication mechanism.

TTV, like the other circular ssDNA viruses, is predicted to use the rolling circle replication system (Erker *et al.* 1999; Mushahwar *et al.*, 1999; Okamoto *et al.*, 2000). Based on the TTV-related circovirus and geminivirus replication system, TTV viral proteins would be required to cooperate with cellular proteins for the initiation of replication (Mankertz *et al.*, 1998; Nigro *et al.*, 1998). For circovirus replication, circovirus encodes replication-associated proteins with specific Rep-motifs which bind to the replication initiation site (Mankertz *et al.* 1998) and, according to the amino acid sequences, TTV ORF1 is predicted to contain similar Rep-motifs (Mushahwar *et al.* 1999; Mushahwar *et al.*, 1999; Tanaka *et al.*, 2001). Animal circoviruses have a conserved rep-protein (replication associated protein) coding sequence and genomic structures (stem-loop structure) which interact for virus replication (Niagro *et al.*, 1998; Mankertz *et al.*, 2001; Mankertz *et al.*, 2001; Todd *et al.*, 2004), and TTVs also contain the similar sequence and form the similar structure (stem-loop structures were located in the GC-rich region and rep-protein was made in ORF1.) (Hijikata *et al.*, 1999; Mushahwar *et al.* 1999; Peng *et al.*, 2002). However, it is still unknown whether these similar structures and proteins are actually functional in the infected cell.

1.5.2. Replication site and Pathogenicity of TTV

TTV DNA can be detected in several organs, indicating a wide range of cell tropism (Reviewed by Hino and Miyata. 2007). Although TTV viral load is higher in bone marrow, lung, spleen, and liver than any other organs in TTV infected individuals, the host cell and details of replication in the cells during TTV infection are still unknown. In addition, virus replication would not necessarily produce a lesion because the cells in which TTV is actively replicating did not show any pathologic change (Cheng *et al.*, 2000; Jiang *et al.*, 2000).

TTV is widespread in general human population so that some researchers suggest that TTV does not have any pathogenic effect. However, TTV, TTMDV, and TTMV have possible disease associations and the lesion of TTV may be shown in the different place from the past studies or require other pathogens to develop. In many cases, viral replication is known to cause cell lysis to spread progeny virions. Viral infection may induce apoptosis to prevent further virus replication, the immune response may destroy infected cells, and viral infection may lead to carcinogenesis. TTV and disease relationships have been described in several cases such as a liver disease (section 1.5.2.1.), respiratory disease (section 1.5.2.2.), multiple sclerosis (MS) (Sospedra *et al.*, 2005), systemic lupus erythematosus (SLE) (section 1.5.2.4.), diabetes mellitus (Guney *et al.*, 2005), laryngeal cancer (section 1.5.2.3.), pancreatic cancer (Tomasiewicz *et al.*, 2005), cancer in general (section 1.5.2.3.), periodontal disease (Rotundo *et al.*, 2004), certain rheumatic disease (section 1.5.2.4.) and CNS disease (Maggi *et al.*, 2010).

1.5.2.1. Virus replication in liver and hepatic disease

As TTV was originally discovered in a hepatitis patient, the liver was the original presumed target organ of TTV replication (Nishizawa *et al.*, 1997). In experimental infection of Rhesus monkeys, viral DNA was observed in various organs, but double-strand circular DNA which

indicates potential replication was detected only in liver, bone marrow, and the small intestine (Xiao et al. 2002). The double stranded circular genome was also observed in human liver (Okamoto *et al.*, 2000). Furthermore, high TTV titer was found in bile (Ukita *et al.*, 1999; Itoh *et al.*, 2001), and TTV can be detected by *in situ* hybridization method in nucleus and/or the cytoplasm of liver cells in patients who have liver disease (Cheng *et al.*, 2000; Jiang *et al.*, 2000). However, TTV infected cells did not show any pathological change. In recent years, there has been increasing recognition that the liver is most likely not the main replication site of TTV (Viazov *et al.*, 1998; Kikuchi *et al.*, 2000; Nakagawa *et al.*, 2000). The double stranded replication form of TTV genomic material has also been found in almost every organ; lung (Bando *et al.*, 2001), stimulated peripheral blood mononuclear cells (PBMC) (Mariscal *et al.*, 2002), bone marrow (Okamoto *et al.*, 2000b), liver (Okamoto *et al.*, 2000a), lymph node, thyroid gland, spleen, pancreas, and kidney (Okamoto *et al.*, 2001). About these results, some researchers proposed that TTV is present in immune cells and they might migrate into the various organs by using these cells. Thus, TTV replication mechanism has to be carefully studied (Takahashi *et al.*, 2002).

As the discovery of TTV from a patient with non-A to non-E hepatitis (Nishizawa *et al.*, 1997), TTV research has concentrated on liver disease and many studies focused on connecting TTV and liver disease such as non-A to E hepatitis, transfusion associated hepatitis, cryptogenic chronic liver disease (Ergünay *et al.*, 2008), liver cirrhosis (Zein *et al.*, 1999), hepatitis-associated aplastic anemia (Ishimura et al. 2010), fulminant hepatitis (Shibata *et al.*, 2000), hepatocellular carcinoma (Tokita *et al.*, 2002) and alcohol related liver disease (Tokita *et al.*, 2001).

Early studies of TTV concentrated on establishing the relationship between TTV prevalence/virus titer and various hepatic diseases (Charlton *et al.*, 1998; Ikeda *et al.*, 1999; Kanda *et al.*, 1999;

Nishizawa *et al.*, 1997; Okamura *et al.*, 2000; Tanaka *et al.*, 1998). It has been suggested that TTV is a potential cause of liver damage in children (Nobili *et al.*, 2005) and persistent TTV infection in the patient could cause hepatic failure (Takayama *et al.*, 1999). Furthermore, TTV hepatitis-associated aplastic anemia was also reported in a 12 year old Japanese boy (Ishimura *et al.*, 2010). TTV related liver disease was also studied by experimental infection using chimpanzees. A serum sample from an 11-month-old infant with acute hepatitis which contained TTV genotype 1 (10^5 copies/ml) was inoculated into a naive chimpanzee intravenously. The chimpanzee showed several symptoms; a histological change (ballooning degeneration of hepatocytes), increasing TTV DNA at 5-15 weeks after inoculation, an elevation of the serum α -glutathione-S-transferase level, and mild elevation of ALT level (Tawara *et al.* 2000). Subsequently, TTV DNA level decreased depending on immunoglobulin (Ig)M-class and IgG-class anti-TTV (genotype 1) antibody suggesting TTV genotype 1 has hepatitis inducing capacity. However, co-infection of other pathogens or host species difference between human and chimpanzee could have influenced this study and also the number of samples is too low to indicate the TTV related disease. In addition, genotype 1 TTV protein may be pathogenic in liver cells. TTV has an Apoptin-like protein (Apoptin is the main apoptosis-inducing agent of CAV) called TTV-derived apoptosis-inducing protein (TAIP), and it is selective to induce apoptosis in hepatocellular carcinoma (Kooistra, *et al.* 2004). These diseases were more frequently observed with Human TTV genotype 1. Hence, several studies suggested that human TTV genotype 1 has hepatitis inducing capacity and may be more pathogenic than other genotypes of TTV (Okamoto. 2009). However, contradictory results have been reported and indicate that TTV is not associated with ALT levels with any form of hepatitis (Hijikata *et al.*, 1999; Hsieh *et al.*, 1999; Nakano *et al.*, 1999; Naoumov *et al.*, 1998; Niel *et al.*, 1999; Prati *et al.*, 1999; Viazov *et al.*, 1998; Yamamoto *et al.*, 1998). Therefore, in current situation, it has been found that the higher level of TTV viremia is observed in hepatitis patient (Pistello *et al.*, 2001), but it was not clear whether

this high TTV viremia is a cause or a consequence of hepatitis (Nobili *et al.*, 2005).

Other candidate genotypes of TTV related hepatitis disease are genotype 12 and 16, which are described as SENV-D and SENV-H respectively. These two genotypes have been more frequently found in transfusion-associated non-A to -E hepatitis patients than among the transfused patient without hepatitis (Umemura *et al.*, 2001). However, some contradicted papers were also published and they indicated that SENV infection is not related to hepatitis and other liver disease (Akiba *et al.*, 2005; Kao *et al.*, 2002; Schroter *et al.*, 2003).

Most studies have not described a significant role for TTV in hepatitis. Hence, co-infection with other hepatitis viruses is another approach in TTV hepatitis research. Co-infection study has been focused on HCV and HBV infections due to the original assumption of TTV as a hepatitis virus. In the chronic stage of TTV infection, the patients infected with HCV and TTV showed a higher histological change compared to the patient infected with HCV only (Sampietro *et al.*, 2001). In the hepatitis B patients, there were no significant differences in ALT level or HBV titer between TTV infected and noninfected children, but the total histological score of disease severity was significant higher in TTV infected patients (Kasirga *et al.*, 2005). However, there is still no clear proof that TTV is a co-factor, disease maker or complicating factor. Also, many papers mentioned that co-infection does not have any effects on hepatitis and other diseases (Campo *et al.*, 2000; He *et al.*, 2003; Hsu *et al.*, 2003; Kato *et al.*, 2000; Schroter *et al.*, 2003; Tuveri *et al.*, 2000; Umemura *et al.*, 2001).

In conclusion, TTV related liver disease has been well studied because of the discovery of TTV in a hepatitis patient. However, these hypotheses have not been supported by further investigation even by the same group of researchers. Overall, TTV is almost ruled out as an important cause of

clinical liver disease.

1.5.2.2. Virus replication in respiratory tract and pulmonary disease

TTV replication is considered to occur in the respiratory tract. TTV replication in epidermal cells of entodermal origin has also been suggested because high TTV viral titer was shown in nasal secretions (Maggi *et al.*, 2003; Deng *et al.*, 2000). In addition, it has been suggested that TTV infection has a potential role in pulmonary disease, because TTV replication was observed in pulmonary epithelium of 1 to 24-month-old children with acute respiratory disease, and group 4 TTV was frequently observed in bronchopneumonia patients (Bando *et al.* 2001; Maggi *et al.* 2003a; Okamoto *et al.*, 2001). It was reported that high TTV loads were found in the swabs of severe acute respiratory disease patients and the prevalence of SAV showed the same result (Chung *et al.*, 2007). Interestingly, active TTV replication was observed in the respiratory tract. This TTV replication is predicted to be related to the lymphocyte imbalance (high B cells and low CD3⁺ and CD4⁺ T cells) (Maggi *et al.* 2003b; Maggi *et al.* 2004) and might skew the immune response towards a Th2 response (Pifferi, *et al.* 2005). The imbalance of Th2 response is thought of as critical to the pathogenesis of asthma (Umetsu *et al.* 2002). In addition, in children with asthma, the three important lung functions (force expiratory flow; FEF in which 25% and 75% of force vital capacity (FVC) is expired (FEF_{25%-75%}), force expiratory volume (FEV), and FEF_{25%-75%}/ FVC) decreased and inversely correlated with nasal viral load. This might indicate that TTV affects lung function. Bando *et al.* reported that idiopathic pulmonary fibrosis (IPF) may be related to TTV infection because TTV infected IPF patient showed poor survival rate and there was an association with lung cancer development in IPF patients (Bando *et al.* 2001; Bando *et al.* 2008). In one study, a newborn showed benign viral rhinitis with TTV infection. The oral swab samples collected in the several times from mother, father, and new born child, and TTV was detected from mother only before the date of newborn delivery. In day 4, new born child showed

TTV infection and the TTV sequence was identical to that of mother samples of 25 days before the newborn delivery. Accordingly, the virus was predicted to come from mother which had high TTV load in saliva (Biagini *et al.*, 2003).

1.5.2.3. Cancer

There are several reports investigating the association between TTV infection and cancer. These are primary focused on four major cancers; hepatocellular carcinoma (HCC) (Michitaka and Onji 2003), lymphoma (zur Hausen and de Villiers 2005), laryngeal carcinoma (Szladek *et al.* 2004), and pancreatic cancer (Tomasiewicz *et al.*, 2005).

Due to the discovery of TTV in a hepatitis patient, it was thought that it may account for the 5 to 10% HCC patients who are hepatitis B virus (HBV) and hepatitis C virus (HCV) negative. In the HBV carcinogenesis, HBV DNA is known to integrate into the hepatocyte DNA during persistent infection and this is involved in the process of hepatocarcinogenesis. TTV was also predicted to show the similar mechanism, but TTV DNA was confirmed not to integrate into host hepatocyte DNA (Yamamoto *et al.* 1998). This would indicate that TTV may use a different mechanism from HBV and HCV. As an alternative, TTV may have some role in carcinogenesis in HBV or HCV infection (Tokita *et al.* 2002).

The etiology of some lymphomas and leukemias is still unknown and chromosomal translocations have been identified in these tumour cells. As the cause of chromosomal translocation, infectious agents have been suspected as the factors related to lymphomas and leukemias development. Epstein-barr virus (EBV), human herpes virus type 8, and human T-cell Lymphotropic virus (HTLV-1) have been reported as viruses inducing lymphomas and leukemias (zur Hausen and de Villiers. 2005). In the TTV studies, TTV DNA was frequently found in

various types of lymphoma and it is suggested that TTV may contribute to a number of lymphoproliferative disorders (Garbuglia et al. 2003). Furthermore, TTV-like viruses were found in 2 cell lines of lymphatic origin and they were Epstein-barr virus (EBV) negative (zur Hausen and de Villers. 2005). Based on this finding, it is assumed that TTV-like virus increases the risk of chromosomal translocation, and this risk would be increased depending on the viral load (zur Hausen and de Villers. 2005).

It is also reported that laryngeal carcinoma patients showed co-infection of genotype 1 TTV and human papillomavirus. The patients infected either human papillomavirus or TTV did not show the papilloma with malignant transformation. Therefore, co-infection of genotype 1 TTV and human papillomavirus might cause poor clinical outcome in laryngeal carcinoma (Szladek et al. 2004).

In another study, two hepatitis patients developed pancreatic cancer and serological and virological markers were used for screening the causative factors. However, only TTV was detected in their serum and it is predicted that TTV was the only detectable causative agent for this cancer development. However, it was very difficult to confirm the relationship between TTV and pancreatic cancer (Tomasiewicz *et al.*, 2005).

Furthermore, TTV may be associated with the development of classical Kaposi's sarcoma. Human herpes virus 8 infection is necessary but not sufficient for the development of Kaposi's sarcoma. TTV infectious rate in the sera of patients with Kaposi's sarcoma was much higher than that in sera of healthy patient. In addition, TTV DNA was detected in cutaneous samples from Kaposi's sarcoma but not observed in control sample from the healthy skin. It was predicted that TTV may play an important role for the development of Kaposi's sarcoma, for example, immunosuppression and/or sharing the common transmission pathway (Girard *et al.*, 2007).

In cancer patients, 100 times higher virus load was observed in the Peripheral blood mononuclear cells (PBMC) compared to those of healthy controls. PBMCs are thought of as the one of the important predicted replication cells and TTV replication was predicted to be related to cancer development. However, still it is not certain TTV is a cause of cancer or shared among the patients with other pathogen (Zhong *et al.*, 2001).

Many cancers have been reported as being TTV associated but two factors have to be considered in the cancer research of TTV infection. 1) TTV carcinogenesis cases may be caused by tissue inflammation. Cancer frequently induces the inflammation and the inflammation encourages TTV to replicate and mutate (de Villiers *et al.* 2007). It may mislead that TTV is related to carcinogenesis of these tumour. 2) It was uncertain whether the high TTV titer in cancer is the cause or result of cancer development. TTV is predicted to infect highly replicating cells (section 1.5.1.).

1.5.2.4. Autoimmune disorders

Autoimmune rheumatic diseases are predicted to have a complex mechanism for development and it is known that at least some cases may have a viral aetiology. It is thought that molecular similarity of virus proteins and autoantigens can cause T and B cell dysfunctions (Gergely *et al.*, 2006). Early in TTV research, some epidemiological studies were published on the association of rheumatic disease and TTV infection (Hirata *et al.*, 1998). Lower TTV infectious prevalence was observed in the rheumatic arthritis patients compared to those of the control patients with other unselected pathology (Maggi *et al.*, 1999 ; Seemayer *et al.*, 2001), although the contradictory results have also been reported (Hirata *et al.*, 1998).

HTLV I-related retrovirus element (HRES-1/ p28) autoantigen has been suggested as a common

autoantibody-generating epitope in systemic lupus erythematosus (SLE). Anti-HRES-1/ p28 antibody were detected in 21-50% of SLE patients in contrast to normal donors or HIV-infected patients (Perl *et al.*, 1995). HRES-1/ p28 is a human endogenous retrovirus-encoded nuclear protein expressed in a tissue specific manner (Banki *et al.*, 1992) and it resembled the peptides from TTV ORF1 and ORF2. HRES-1/ p28 reactive serum has been shown to recognise at least one TTV peptide in TTV infected patients (Gergely *et al.*, 2005). In addition, TTV titer was much higher in SLE patients than in healthy relatives. Genetic factors are thought to be an important risk factor for SLE and TTV infection may skew the genetic factor of SLE (Gergely *et al.*, 2006). However, it is not clear whether TTV enhances generation of autoantibody or the patients who have a immunological dysfunction tend to be more likely to harbour TTV infection than healthy individuals (Gergely *et al.*, 2005).

Multiple sclerosis is also a candidate TTV related disease. In one study, CD4⁺T cells taken from a patient with multiple sclerosis responded against the poly arginine motif of TTV ORF1 N-terminus. It is predicted that these T cell clones were stimulated repeatedly by arginine rich protein and the T cells starts to recognise multiple autoantigens. Finally, the combination of the stimulation from TTV and other genetic and microbial factors could have a role of disease development (Sospedra *et al.*, 2005). Moreover, TTV has been detected in patients with rheumatoid arthritis (Gergely *et al.*, 2005; Hirata *et al.*, 1998) and idiopathic inflammatory myopathy (IMM) (Gergely *et al.*, 2005). As genotype 4 was frequently observed in the rheumatoid arthritis patients, it is also suggested that the specific genotype such as TTV genotype 4 caused the rheumatoid arthritis (Maggi *et al.*, 2007). However, the elevation of TTV load was not observed in the rheumatoid arthritis patients but was in patients with SLE and psoriatic arthritis (PsA). Finally, the authors suggested that the stimulation of TTV replication and autoimmune reactivity may be related but the actual causality is not confirmed (Maggi *et al.*,

2007).

1.5.2.5. Virus replication in bone marrow cells and haematological disorder

In humans, double stranded circular TTV DNA is found in the bone marrow of infected individuals suggesting TTV replication (Okamoto *et al.* 2000). TTV has also been detected in granulocytes (Takahashi *et al.* 2002) and TTV replication was observed in phytohaemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMC) in humans (Mariscal *et al.* 2002; Maggi *et al.* 2001) although virus replication was only for a short period of time and the copy numbers were low. This virus replication in PBMC was also confirmed by fluorescent *in situ* hybridization (Lopez-Alcorocho *et al.*, 2000; Mariscal *et al.*, 2002; Zhong *et al.*, 2002) and it has also been speculated that virus heterogeneity may cause a preference for PBMC or other organs (Chan *et al.*, 2001; Okamoto *et al.*, 1999; Okamoto *et al.*, 2000). Also, the erythroid lineage may be ideal for TTV replication, because the promoter activity of TTV was strongest in K562 cells of erythroid origin (Kamada *et al.*, 2004). Cells of the megakaryocyte lineage could also be a possible replication site as thrombocytopaenia was reported in TTV infected patients (Tokita *et al.*, 2002). In patients under myelosuppression, TTV DNA expression levels were decreased suggesting that TTV was sustained by bone marrow cells (Yu *et al.*, 2002; Zhong *et al.*, 2002), and TTV was also cleared after bone marrow transplantation (Chan *et al.*, 2001) However, it is also possible that unidentified cells may be the host of TTV, or TTV different genotypes could show different host cell tropism.

As TTV replication in bone marrow cells has been shown, links between individuals and haematological disorders have also been studied well. Hepatitis-associated aplastic anemia (HAAA) is known to occur after non-A, non-B and non-C hepatitis (Brown *et al.*, 1997). TTV infection has been suggested as a contributory factor because a high level of TTV replication was

observed in the bone marrow of the HAAA patients (Kikuchi *et al.*, 2000; Ishimura *et al.* 2010; Miyamoto *et al.*, 2000). However, contradictory reports have also been published showing no TTV detection in HAAA patients in the epidemiological study (Poovorawan *et al.*, 2001; Safadi *et al.*, 2001). As previously discussed in section 1.2.3., TTV DNA was detected in the lymph nodes of patients with B-cell lymphoma and those with Hodgkin`s disease (Garbuglia *et al.*, 2003). It is predicted that TTV may affect the infected T cells and play the role of lymphoma development.

1.5.2.6. Co-infections with other pathogen

It is hypothesized that co-infection is another important factor for the development of TTV associated disease. Co-infection with human papillomavirus (HPV) was frequently observed in TTV infected women patients, and the patients with HPV infection showed higher TTV titer, although no cancer development was observed (Calcaterra *et al.*, 2001). Also, in the case of laryngeal cancer, TTV genotype 1 and HPV co-infected patients showed the malignant transformation of papillomatosis and the poor clinical outcome (Szladek *et al.*, 2005). Following the report, oral squamous cell carcinoma and cervical cancer were studied and showed the similar result (Feher *et al.*, 2009). Higher TTV loads were observed in the gastritis patients with *Helicobacter pylori* and it is suggested that TTV may have some role in the development of gastritis (Maggi *et al.*, 2003).

1.5.2.7. Immunocompromised patients

It is predicted that TTV viral titer may depend on the immunological status of host. HIV infected patients are more likely to have some TTV genotypes compared to the healthy individuals (Shibayama *et al.*, 2001). HIV positive patients receiving highly active antiretroviral therapy (HAART), decreased TTV virus titer probably because of the recovery of immune system even in

the absence of CD4⁺ T cell number improvement (Madsen *et al.*, 2002). Other studies have shown that TTV viral load is increased in HIV patients progressing toward acquired immunodeficiency syndrome (AIDS) and it was related to a low CD4⁺ cell count (Christensen *et al.*, 2000; Sagir *et al.*, 2004; Sagir *et al.*, 2005; Shibayama *et al.*, 2001; Thom *et al.*, 2007; Touinssi *et al.*, 2001). However, these studies have not established the consequence of TTV infection on HIV disease progression.

1.6. Immunology of TTV

Anellovirus spread in a variety of host species and show wide genetic diversity (section 1.1.4. and 1.2.). These facts indicate that anelloviruses have established a highly successful interaction with the host immune system and have thrived in the hosts leading to the presence of numerous genotypes. However, because of the lack of the investigational tool including sufficiently efficient *in vitro* cell culture system and easy-to-handle experimental animals (section 1.7.), anellovirus immunological properties are still poorly understood. Although the information of anellovirus related host immunological reactions are limited, we review the recent studies of the innate, adaptive and cell-mediated immunity.

1.6.1. Innate immunity

The first possible defense system of TTV infection is predicted to be the ciliated cells in the respiratory epithelium because the ciliated cells have non-specific defence function of continuously sweeping pathogens by using mucous secretions. It was shown that the respiratory tract, especially the upper tract, was continuously exposed to TTV and is predicted to be an important route and primary replication site of TTV infection (Maggi *et al.*, 2003). The cell type of TTV replication has not shown but the ciliated cells showed the abundant TTV titers according to the study by using laser micro-dissected epithelial cells. Furthermore, it was shown that high

TTV viral load was related to the disfunction of the "mucociliary escalator" system (Pifferi *et al.*, 2008). This may indicate that TTV disturbed the defense system of respiratory tracts.

Specific TTV proteins may function to disturb the host immune system. ORF2 of genotype 3 TTV SANBAN strain was shown to have the potential to interfere with the activity of NF- κ B. NF- κ B is well-characterised intracellular signal transcriptional factor and NF- κ B activity promotes virus related inflammation (Zheng *et al.*, 2007).

TTV infection is a suspected cause of asthma and acute respiratory disease (section 1.5.2.2.). TTV viral load had a positive correlation with the concentration of Eosinophil Cationic Protein (ECP) in children with acute respiratory disease (Maggi *et al.*, 2004) and TTV load in nasal fluid and exhaled Nitric Oxide (eNO) also showed the positive relationship (Maggi and Bendinelli, 2009). ECP and eNO have a complex function related to respiratory disease and have a key role of respiratory inflammation (Maggi *et al.*, 2004; Pifferi *et al.*, 2011). ECP is thought to be a reliable indicator of the inflammation in the clinics. Moreover, these immunological disturbances would be directly connected to the virus replication and produce the abundant virus-immune complexes.

1.6.2. Adaptive Immunity

Antibodies against TTV were detected using various methods. When TTV from faecal supernatants and the patient serum were mixed together, immunoprecipitation was observed. These precipitated pellets showed higher TTV PCR signal and the presence of TTV-specific antibodies were confirmed in the serum. This study showed that regardless of the presence of TTV DNA or not, the patients could have anti-TTV IgG antibodies (Tsuda *et al.*, 1999).

In spite of the many limitations of TTV research, there are several attempts to reveal the kinetics

of antibody production in primary infection. Sequential examination of the patients who were accidentally infected with TTV and chimpanzees used in TTV infectious experiments showed that the antibody elevation was slow similar to hepatitis C virus infection (Tsuda *et al.*, 1999; Tsuda *et al.*, 2001; Tawara *et al.*, 2000; Ott *et al.*, 2000). TTV-specific IgM was detected in serum 10-21 weeks after the inoculation. This was 2-7 weeks after the appearance of TTV DNA in blood. Usually IgM decreased within 5-11 weeks (Tsuda *et al.*, 2001; Kakkola *et al.*, 2002). IgG appeared after IgM and lasted longer to retain a relatively constant titer. Most of these results came from TTV genotype-1 infection, and it is uncertain whether TTV genotype influences the serotypes produced, kinetics, and cross-reactivity after antibody response.

The relationship between TTV ORF1 and ORF2 proteins and autoimmune disease was discussed in section 1.5.2.4.. Kakkola *et al.* (2002) provided the evidence to support TTV ORF2 immunogenicity. TTV ORF2 can be divided into ORF2a and ORF2b (section 1.4.3.) and these two proteins can be produced separately by using the prokaryotes. The presence of anti-ORF2 antibodies in the sera of 89 healthy individuals was screened using these two ORF2 proteins, and IgM and IgG were detected in 9% and 10% of samples respectively. In a subsequent study, Gergely *et al.* (2005) found anti-ORF2 IgG in nearly 90% of SLE patients. These results indicate that TTV ORF2 is immunogenic and could be associated with the development of autoimmune disease as discussed in section 1.5.2.4.

The putative TTV capsid protein, ORF1 was also expressed by two research groups, with one group succeeding with production of the N-terminus (amino acid 1-411) (Handa *et al.*, 2000) and the other group reporting the expression of the C-terminus (Ott *et al.*, 2000). Both proteins were expressed as His-tagged fusion in *E. coli*. and the proteins were used to detect TTV specific antibody. The antibodies against N-terminus of ORF1 were detected in 38% U.S. blood donor

and the majority of antibody positive individuals were TTV DNA positive suggesting that the antibodies were not effective in preventing TTV infection or may be a marker of chronic infection (Handa *et al.*, 2000). On the other hand, antibodies (IgG+IgA+IgM) against the C-terminus of ORF1 were detected in 98.6% of sera. In the same samples, TTV-DNA was found in 76.1% (Ott *et al.*, 2000). However, no antibodies were detected by using the protein from N22 region of ORF1 (120 amino acid) indicating that the immunogenesis may be region dependent (Lo *et al.*, 1999).

TTV particles containing immune complexes were observed in the sera of infected individuals (Itoh *et al.*, 2000; Maggi *et al.*, 2006; Tsuda *et al.*, 1999) and the number of virions decreased every day (Maggi *et al.*, 1999). These immune complexes were common in chronic viral infections such as HCV and HIV, however in acute infection these immune complexes were not observed (Nishizawa *et al.*, 1999). It has been reported that superinfection including a genogroup 2 TTV (ViPi04 strain which is uncommon strain in genogroup 2) and three different genotypes of TTV developed the rapid increase of total plasma viremia and the decrease of immunocomplexes in TTV infected patients. After superinfection, the immune complexes were recovered and it may suggest that the antibody response is effective in the acute phase of TTV infection (Maggi *et al.*, 2006).

1.6.3. Cell-mediated immunity

It has been shown that there is a relationship between high TTV viral load and immune suppression in individuals such as the transplant recipients (Moen *et al.*, 2003) and HIV infected patients (Sagir *et al.*, 2005a; Touinss *et al.*, 2001). CD4⁺ T cell is predicted to be inversely correlated with TTV titer and the patients with lower CD4⁺ T cell count showed higher TTV level (Christensen *et al.*, 2000; Sagir *et al.*, 2005b; Thom *et al.*, 2007) indicating that TTV viral load

seems to influence, or be influenced by, host immune status (Madsen *et al.*, 2002; Sagir *et al.*, 2005a; Shibayama *et al.*, 2001). Similar balance of TTV and lymphocytes was observed in children with acute respiratory disease (section 1.5.2.2.) and in this disease, high B cell number was positively related to TTV viral load but T cell number was decreased (Maggi *et al.* 2003b; Maggi *et al.* 2004). Patients with HIV related immunodeficiency alone did not show a significant TTV and TTMV elevation and it was not correlated to the number of CD4⁺ cells. Furthermore, the patients who had HAART (highly active antiretroviral therapy) decreased TTV viral load even without increase CD4⁺ cell number indicating that the other immunological mechanism may influence the viremia (Madsen *et al.*, 2002; Moen *et al.*, 2002).

Interferon is widely used for the treatment of chronic hepatitis B and C infection. The effects of interferon treatment on TTV infection were also studied, and several studies showed that TTV and TTMV level decreased depending on the interferon treatment regimen indicating a suppression of TTV replication (Akahane *et al.*, 1999; Ali *et al.*, 2002; Chayama *et al.*, 1999; Kao *et al.*, 2003; Maggi *et al.*, 2001; Tokita *et al.*, 2001; Umemura *et al.*, 2002; Watanabe *et al.*, 2000). Interferon was thought not to eradicate TTV because genetically identical virus reappeared in the patients following removal of interferon treatment. Furthermore, novel strains also appeared after the removal of interferon treatment indicating re-infection (Moen *et al.*, 2003). It was also predicted that some specific genotypes are more resistant to interferon treatment (Chayama *et al.*, 1999).

1.7. Model of TTV infection

1.7.1. Cell culture system of TTV

One of the major TTV research problems is the lack of a suitable cell culture system. Although various cell lines and primary cells have been tested for their ability to support TTV replication,

TTV infection in these cells was not sufficient to reliably produce infectious virus. Reproductive form of TTV was found in bone marrow, peripheral blood mononuclear cells (PBMC) and liver cells (section 1.5.2.1.). As a result, the cell culture study has concentrated on using haematopoietic cell lines and liver cell lines. In the early experiment, the PBMC from TTV-DNA negative healthy individual was used in an *in vitro* experiment. In an experiment comparing stimulated PBMC and unstimulated PBMC, TTV-DNA and mRNA were detected in only stimulated PBMC (Mariscal *et al.* 2002). Accordingly, it was demonstrated that TTV could replicate in stimulated PBMC (Maggi *et al.*, 2001; Mariscal *et al.* 2002), although virus titer is low. The liver also had been thought as the main site of TTV replication because TTVs were originally discovered from hepatitis patient (Nishizawa *et al.*, 1997). It was reported that the clear morphological change such as clumping and granular degeneration was observed in the Chang Liver cell line (Desai *et al.* 2005) upon infection with TTV. TTV replication was seen in the Raji cell line, B-cell line (Desai *et al.*, 2005), 293 cell human embryonic kidney, Huh7 hepatocarcinoma cell line, and UT7/Epo-S1 myeloid cell line; (Kakkola *et al.* 2007) but these studies showed only low-level infectivity. Also, these TTV replications stopped within a short period of time and virus propagation was not observed in these reports (Mariscal *et al.* 2002., Desai *et al.* 2005., Kakkola *et al.* 2007., Leppik *et al.* 2007). Recently, human TTV subviral molecules were observed in the medium of TTV complete genome transfected 293TT cells and the subviral molecules transfected 293TT cells also produced the subviral molecules (de Villiers *et al.*, 2011). However, these subviral molecules were not infectious and the copy number decreased over time (de Villiers *et al.*, 2011).

1.7.2. Animal model of TTV

Another difficulty in TTV research has been the lack of a suitable animal model. Animal experimentation in TTV has been conducted in chimpanzee, pig and transgenic mouse.

The first attempt to make an animal model of TTV infection was using chimpanzees. In this study, human TTV genotype 1 from human faecal supernatant or serum was inoculated intravenously into chimpanzees and they showed mild biochemical and histological change in liver (Tawara et al. 2000).

The best animal model to date has been the infection of pigs. The international Committee on taxonomy of virus (ICTV) has grouped TTVs in nine genera depending on the host species. There are currently 2 recognised genera of anellovirus that infect pigs; *Torque teno sus virus 1* (TTSuV1), genus *Iotatorquetenovirus* and *Torque teno virus 2* (TTSuV2), genus *Kappatorquetenovirus*. Porcine TTV also showed the high genetic diversity similar to human TTV infection and one individual can have at least two genotypes or subtypes in the same species (Gallei et al., 2010; Huang et al., 2010). TTSuV1 and TTSuV2 shared very low level of nucleotide identities about 50% (Huang et al., 2010; Cortey et al., 2011) and even in the same species, TTSuV1 and TTSuV2 showed high intraspecies variation (>30% in TTSuV1 and >15% in TTSuV2) (Huang et al., 2010; Cortey et al., 2011). The epidemiological character of TTSuV infection is similar to that of human TTV infection. TTSuV infection has been detected in swine serum worldwide and its infectious rate ranged from 24% to 100% (McKeown et al., 2004; Bigarre et al., 2005; Kekarainen et al., 2006; Taira et al., 2009; Gallei et al., 2010). Transmission routes are considered to be same as other TTV; faecal-oral and vertical transmission and early acquisition of TTSuV was observed from one year old (Martinez-Guino et al., 2009; Martinez-Guino et al., 2010; Pozzuto et al., 2009; Sibila et al., 2009).

Pigs inoculated intraperitoneally with porcine TTV genotype 1 serum showed histological change such as interstitial pneumonia, transient thymic atrophy, membranous

glomerulonephropathy and modest lymphocytic to histiocytic infiltrates in the liver (Krakowka *et al.*, 2008a; Krakowka *et al.*, 2008b).

Most TTSuV infected pigs are apparently healthy (Sibila *et al.*, 2009b), but it is believed that TTSuV can be one of the key factors in the development of some diseases. Co-infection with PCV-2 has mainly been studied and TTSuV is considered to be related with the occurrence of porcine circovirus associated disease (PCVDs) such as post-weaning multisystemic wasting syndrome (PMWS) (Aramouni *et al.*, 2011; Ellis *et al.*, 2008; Kekarainen *et al.*, 2006; Nieto *et al.*, 2011) and a porcine dermatitis and nephropathy syndrome (PDNS)-like condition (Krakowka *et al.*, 2008). TTSuV 2 viral titer was significantly higher in PMWS compared to healthy or PDNS infected pigs, but TTSuV 1 viral titer showed no difference between PCV-2 infected pigs and healthy pigs (Aramouni *et al.*, 2011). However, the contradictory results have shown that TTSuV were not involved in PCV-2 related disease (Gauger *et al.*, 2011; Lee *et al.*, 2010). Also, TTSuV 1, but not TTSuV 2 was detected frequently from porcine respiratory disease complex (PRDC) affected pigs and it was concluded that TTSuV was strongly related to clinical multietiological condition (Rammohan *et al.*, 2012).

In addition to its role in co-infection, there were several attempts to reveal the pathogenesis of TTSuV single infection. Krakowka and Ellis (2008) showed the development of mild lesions by inoculating TTSuV containing plasma into gnotobiotic pigs. TTSuV infected pigs showed transient thymic atrophy, membranous glomerulonephropathy, modest lymphohistiocytic infiltrates in the liver and mild interstitial pneumonia (Krakowka and Ellis. 2008). Similar results were also observed in TTSuV2 infectious experiment by inoculating liver homogenate with TTSuV2 into specific-pathogen free pigs (Mei *et al.*, 2011). Recently, a new approach was carried out using the porcine TTV infection system. In this method, porcine TTV concatemerised

DNA was directly inoculated into the piglets and virus replication was observed (Huang *et al.*, 2012). By using this method, TTV was not needed to be isolated in the cell culture and it is expected to be used in pathogenesis and biology of TTVs.

Because of the lack of cell culture system (section 1.7.1.), TTVs have not been isolated and these experiments were performed by using the serum, faecal supernatant and liver homogenate (Tawara *et al.* 2000; Krakowka and Ellis. 2008; Mei *et al.*, 2011). Also, even though injection of concatemerised TTSuV2 genome worked *in vivo*, it did not work for *in vitro* studies (Huang *et al.*, 2012).

The transcription profiles of TTSuV were also studied and the protein expression was performed. TTSuV 1 showed two transcripts encoding three predicted proteins and TTSuV 2 had two transcripts encoding six predicted proteins using *in vitro* transfection of TTSuV complete genome (Martinez-Guino *et al.*, 2011; Huang *et al.*, 2011). These predicted proteins were expressed in the porcine kidney cell lines. ORF1 and ORF3 expressions were located in the nucleoli and ORF2 was expressed in the cytoplasm and nucleus excluding the nucleoli. These expressions were similar to those of the homologous proteins from human TTV (Martinez-Guino *et al.*, 2011).

To gain the ideal animal model, there was an attempt to infect mice with human TTV. Generally, human TTV does not infect mouse, and as a result, Tawara and coworkers produced the transgenic mice which contained TTV ORF1. These mice developed renal failure with severe renal epithelial cells abnormalities (Yokoyama *et al.* 2002).

TTSuV is widely distributed in pig population and the body size and physiology of the pig are relatively similar to those of the human (Lunney *et al.*, 2007). As a consequence, it is believed

that the pig may provide a suitable animal model system in TTV research (Kekarainen and Segales 2009). However, a mouse animal model would be more useful because the mice are small, generally easy and economic to maintain and breed prolifically. In addition, there are many reagents and transgenic to study pathogenesis in mice. Human TTV infection in mice is difficult and there is only one report but its infection was only short period time (Isaeva and Viazov, 2002).

1.8. Project outline

Recently, in our laboratory, rodent TTV was found in wood mouse by using RCA. In this project, I aim to investigate the possibility of rodent TTV as a human TTV animal model to study the pathogenesis of TTV. I would like to focus on the following areas.

- (1) Characterisation of rodent torque teno virus in wild rodent population
- (2) Transcripts identification and protein expression of rodent torque teno virus
- (3) Torque teno virus replication *in vitro*
- (4) *In vivo* experiment of Torque teno virus infection in wood mice and type I IFN receptor KO mice

CHAPTER II. MATERIALS AND METHODS

2.1. Tissue samples

2.2. DNA extraction

2.3. RNA methods

2.4. Reverse transcription of RNA

2.5. Polymerase chain reaction (PCR)

2.6. RFLP (Restriction Fragment Length Polymorphism) for identification of species

2.7. PCR product column purification and gel extraction

2.8. Sequence

2.9. Molecular cloning

2.10. Construction of DNA libraries

2.11. Development of assay for gene expression analysis using quantitative real-time polymerase chain reaction (real-time-qPCR)

2.12. Rapid amplification of 5' and 3' cDNA ends (RLM-RACE)

2.13. Protein expression of RoTTV transcripts

2.14. Western blot

2.15. *In vitro* experiment

2.16. *In vivo* transfection

2.1. Tissue samples

Wild rodents (n=266) captured by humane traps in the United Kingdom, and laboratory mice (n=3) were euthanized by cervical dislocation. These rodents were provided by Anna Meredith and Darren Shaw (R(D)SVS, University of Edinburgh)/James Stewart (University of Liverpool). In addition, pet shop feeder dead mice (n=25) which were sold as snake food were used for the samples. Sixty-four spleen and 208 liver samples were taken from wild mice, and 4 spleen and 28 liver samples were obtained from laboratory mice and feeder mice respectively.

2.2. DNA extraction

2.2.1. DNA extraction

Spleen and liver samples were digested by Proteinase K (final concentration 2mg/ml) in 10 volumes of lysis buffer (4% SDS, 0.5M Tris, 0.25M EDTA, 2.5M NaCl) at 53 °C overnight. Then, DNA purification was performed. Firstly, the digested samples were added to an equivalent volume of phenol:chloroform:isoamyl alcohol (IAA) (50:49:1) and vortexed vigorously. Secondly, the mixture was centrifuged >15,000xg for 4 minutes to separate the organic and aqueous phase. The aqueous phase was transferred into a fresh 1.5ml microcentrifuge tube (Eppendorf, UK) and repeatedly extracted with phenol:chloroform:IAA until no protein was visible. Thirdly, the aqueous phase was totally mixed and purified with the chloroform only and centrifuged >15,000xg for 4 minutes and carefully removed into a fresh 1.5ml microcentrifuge tube (Eppendorf, UK). Finally, the DNA was concentrated by ethanol precipitation. The aqueous phase in the 1.5ml microcentrifuge tube (Eppendorf, UK) was added to 1/10 volume of 3M sodium acetate (pH5.2) and 2.5x volume of cold 95% ethanol (v/v), mixed and precipitated at -20°C for at least 2 hours and the mixture was centrifuged at >14,000xg for 30 minutes. The supernatant was discarded and the DNA pellet was washed with 70% (v/v) ethanol and resuspended in DNase-RNase free water.

In order to extract DNA from faeces, DNA was extracted by using QIAamp[®] DNA Blood Mini kit (QIAGEN). Faeces were mixed with PBS and centrifuged at 15,000xg for 5 minutes. The supernatant was taken for DNA extraction. DNA extraction was performed following the manufacturer's instructions. Briefly, 20µl proteinase K, 200ul faecal supernatant 200AL Buffer were mixed together and incubated in 56°C 10 minutes. After that, 200µl 96-100% ethanol (v/v) was added to the digested samples and these sample mixtures were applied in QIAamp spin column and centrifuged at 6000xg for 1 minute. QIAamp spin column was washed by using AW1 and AW2 Buffer. DNA was eluted by using 50µl AE buffer.

2.2.2. Quantification of DNA

DNA quantification was performed by the Nanodrop ND-1000 spectrometer (Nanodrop Technologies, Inc). A 2µl aliquot of distilled water was used for blank measurement. Then, 2µl DNA sample was applied to measure DNA concentration and purity. For screening PCR analysis, DNA was diluted with DNase-RNase free water to give a concentration of 250 mg/ml.

2.3. RNA methods

2.3.1. Tissue collection and storage

For RNA work, wild wood mice were captured by humane trap and euthanized by cervical dislocation. They were dissected and lung, kidney, liver, spleen, and bone marrow were taken. These tissues were cut finely and fixed in RNA later[™] (Ambion) 4°C overnight. After that, these tissues were stored in -20°C freezer.

2.3.2. RNA extraction

DNA and RNA from infected wild mice were extracted by using All Prep mini kit (QIAGEN) according to the manufacturer's instructions. Briefly, up to 30mg tissue was homogenised using a

sterile plastic pestle (Sigma) in Buffer ALT. The viscous lysates were put into an All prep DNA spin column and centrifuged for 30s at $\geq 8000 \times g$ ($\geq 10,000$ rpm).

For the RNA extraction, 96-100% ethanol (v/v) was added to the flow-through from the previous step, applied to an RNeasy spin column, and loaded on the column by centrifugation. Then, the RNeasy spin column was washed with using RW1 and Buffer RPE. After RW1 wash, on-column DNase digestion was carried on. Finally, 50 μ l RNase-free water was applied in the spin column membrane to elute the RNA.

For the DNA extraction, the All prep DNA spin column washed with 500 μ l of buffer AW1 and AW2. The All prep DNA spin column was then centrifuged at full speed for 2 minutes to remove the buffer. Finally, DNA was eluted by 50 μ l of Buffer EB.

2.3.3 DNase digestion of mouse total RNA using TURBO DNA-freeTM Procedure.

During the RNA extraction, on-column DNase digestion was performed. However, by this method, the TTV genome DNA concentration was too high to be removed completely from mouse total RNA. For this reason, TURBO DNATM DNase (Life technologies, UK.) treatment was used for DNA digestion. In the ABgene 0.2ml strip tube (Thermo scientific, UK.), 8 μ l total RNA, 1-2 μ l 1X TURBO Dnase (2U), and 1 μ l 10X TURBO Dnase Buffer were mixed together and incubated in 37°C 30 minutes. Afterwards, 2 μ l DNase inactivation Buffer was added to remove DNase.

2.3.4. Measurement of quality and quantity of RNA by using Agilent RNA 6000 Nano

RNA quality and quantity was measured by using Agilent RNA 6000 (Agilent Technologies, Inc.) following the manufacturer's protocol. RNA 6000 Nano gel matrix was prepared in new RNA 6000 Nano chip and 1 μ l RNA was loaded in the well. After the horizontal vortex, the chip was

set up and run in the Agilent 2100 bioanalyzer according to manufacturer's instruction.

2.4. Reverse transcription of RNA

cDNA was generated from total RNA using SuperscriptTM III Reverse Transcriptase (Invitrogen). In ABgene 0.2ml strip tube (Thermo scientific, UK.) tube, 8µl of DNase treated RNA was incubated with 50ng random primers and 1µl 10mM dNTP mix in a total volume of 13µl for 5min at 65°C. The mixture was immediately chilled on the ice for 2 minutes. 4µl 5x First-strand buffer, 1µl 0.1M dithiothreitol (DTT), 1µl (40 U) RNase OUT and 1µl SuperScript III RT (200U) was added to the RNA/Primer/dNTP mixture. When random primers were used for RT, the mixture was incubated at the room temperature for 5min. Then, the mixture was incubated at 50°C for 1 hour prior to inactivating the reaction at 70°C for 15 min. Then, 1µl (2U) *E. coli* RNase H was added and the reaction mixture was incubated at 37°C for 20min to remove RNA complementary to the cDNA. For the PCR, 1-2µl cDNA was used.

2.5. Polymerase chain reaction (PCR)

2.5.1. Standard PCR/primer design

Standard PCR were performed using GoTaq DNA polymerase (Promega). Reactions were performed by using 37.2 µl of water, 10 µl of 10x buffer, 1 µl of 10mM dNTP (Promega), 1 µl of 10mM forward and reverse primer, and 0.5 µg samples of extracted nucleic acid as template under the following conditions: 30 cycles of [18 seconds at 94°C, 30 seconds at 50°C and 90 seconds at 72°C] and a final extension of 6 minutes at 72°C. Primer sequences are displayed in Table 2.5.1.

2.5.1.1. Pan-TTV primers used for the screening

The sets of degenerate primers (pan-TTV primer) used for screening TTV infection were

constructed by using the sequence result from the published data (Thom. *et al.*, 2003) and hemi-nested primers were designed based on the three parts of highly conserved UTR sequences. In addition, we modified the primer design to detect RoTTV for screening the wild rodents. These primers are designed to recognise the untranslated region (UTR) which is highly conserved in TTV genome structure and this primer set should detect all currently known TTV. PCR was performed by hemi-nested pan-TTV primer shown Table 2.5.1. PCR condition was followed by standard PCR (section 2.5.1.)

2.5.1.2. Screening PCR using genotype specific primer (ORF1 primer) in wild rodent

Two sets of primer pairs for each rodent TTV genotype were designed based on the complete rodent TTV sequence. These primers should bind sequences in open reading frame 1 (ORF1 primer). PCR reactions for screening TTV were performed using GoTaq DNA polymerase (Promega) (section 2.5.1.) and genotype specific ORF1 screening primer shown Table 2.5.1. PCR condition was followed by standard PCR (section 2.5.1.)

2.5.2. Full genome-length PCR

Amplification of complete genome was carried out using AccuPrimeTM Taq High Fidelity (Invitrogen). Reactions were performed using 2.5 µl of 10x AccuPrimeTM PCR Buffer I, 20.4 µl of water, 0.5 µl of AccuPrimeTM Taq High Fidelity, 0.3 µl of 10mM forward and reverse primer and 1 µl amplified DNA by rolling circle amplification (RCA, section 2.10.2) as template, and the inverted primers shown Table 2.5.1. PCR programme was set up as follows: 30 cycles of 18 seconds at 94 °C for denaturation, 21 seconds at 50 °C and 3 minutes at 72 °C, and in the end, a final extension of 5 minutes at 72 °C was performed.

Target	Orientation	Length	Sequence
PAN TTV Screening Primer (used in 3.2)			
TTV UTR CVOS	Sense Inner	22	5' VRV RTK MCG AAT GGC WGA GTT T 3'
TTV UTR CVOA	Antisense Outer	20	5' WGC CCG ADT TGC CCC TTR MC 3'
TTV UTRCVIA	Antisense Inner	16	5' CCT YCG RYW CCC GCC C 3'
TTV Full-length PCR Primer (used in 3.4)			
RoTTV Gt1a	Sense	29	5' GGG ACC GGG CGA GTA TCT AGC TGC GAA GC 3'
RoTTV Gt1a	Antisense	30	5' CCG GTC TCG CAS AGW AAA CTC WGC CAT TCG 3'
RoTTV Gt1b	Sense	29	5' GGG ACC GGG CGA GTA TCT AGC TGC GAA GC 3'
RoTTV Gt1b	Antisense	28	5' GCT TCG CAG CAA GTT ACT GCC CGG TCC C 3'
RoTTV Gt1c	Sense outer	25	5' CGC AGC AGC GCG TCT ATA AAC CAT C 3'
RoTTV Gt1c	Antisense outer	26	5' CGC TGA TGT GTT ACT GCC CGG TCC CG 3'
RoTTV Gt1c	Sense inner	25	5' CCG GCG GCC TGG GCG GGA ATC GGA G 3'
RoTTV Gt1c	Antisense inner	26	5' CGC AGC AAA AAC TCA GCC ATT CGT CAT CT3'
RoTTV Gt2	Sense	24	5' CGA CGA GAC RMA GTC GGR AAG GAG 3'
RoTTV Gt2	Antisense	31	5' CGT CCT CTG ACA GCA TAA ACT CWG CCA TTC G 3'
Genotype specific ORF1 TTV Screening Primer (used in 3.7)			
RoTTV Gt1 ORF1	Sense outer	21	5' CGH ACC TAC MGA CGM AGA RCT 3'
RoTTV Gt1 ORF1	Antisense outer	24	5' CHA CCA TRT CAA ABC CAC ART TRC 3'
RoTTV Gt1 ORF1	Sense inner	22	5' AST AGR AGA TGT GTK ATC AGA A 3'
RoTTV Gt1 ORF1	Antisense inner	23	5' CCC ATC CAY ARK TCA CTV CCA TC 3'
RoTTV Gt2 ORF1	Sense outer	20	5' TTC GTT ACC CTC GCC GGA AC 3'
RoTTV Gt2 ORF1	Antisense outer	21	5' ACC CTT GGT TGC TTT TGG ACC 3'
RoTTV Gt2 ORF1	Sense inner	19	5' CCG CCG AAG GGT AAG GAG G 3'
RoTTV Gt2 ORF1	Antisense inner	21	5' CTC TCC AGA AYC TCT CTT CCC 3'

Table 2.5.1. Primer pair which were used for TTV screening and amplification of complete genome.

2.5.3. Agarose gel electrophoresis

PCR amplicons were visualised by agarose gel electrophoresis. The agarose gels (2% gel (w/v) for less than 1 kbp DNA and 1% gel (w/v) for more than 1kb and less than 3 kbp DNA) were made by dissolving LE agarose powder (Promega) into TAE buffer and adding SYBR® Safe to stain the DNA (Invitrogen). Then, the product size was determined by visualising and comparing bands and ladder under UV light by using UV-transillumination advanced imaging system.

2.6. RFLP (Restriction Fragment Length Polymorphism) for identification of species

For species identification, degenerate primers were designed based on the rodent mitochondrial cytochrome B sequence (Mt Forward: CACTAYACATCAGAYACAWYAACAGC and Mt Reverse: TARGGGTGRAAYGGRATTTTTRTC). PCR reaction was carried out by using 1 µl DNA extraction samples as template. PCR programme was set up under the following condition: 30 cycles of 18 seconds at 94 °C for denaturation, 21 seconds at 50 °C and 90 seconds at 72 °C, and a final extension of 5 minutes at 72 °C was performed.

To identify the mouse species, suitable restriction enzymes were chosen based on the mouse sequences. House/lab mouse (*Mus musculus*), field vole (*Microtus agrestis*), wood mouse (*Apodemus sylvaticus*) and bank vole (*Myodes glareolus*) sequences were submitted in NEB cutter web site (<http://tools.neb.com/NEBcutter2/>) and RsaI and AccI were found to be suitable. The restriction enzyme reactions were performed in ABgene 0.2ml strip tube (Thermo scientific, UK.) containing 5µl mitochondrial PCR products, 0.5µl restriction enzyme, 3µl NEB buffer 4 and 21.5µl nuclease-free water, and incubated at 37°C 30-45 minutes. PCR amplicon was digested by RsaI to identify house mouse and field vole, and then AccI was utilized to find wood mouse and bank vole.

2.7. PCR product column purification and gel extraction

2.7.1. MinElute® PCR purification kit

DNA digested by restriction enzymes, PCR amplified products, and T4 ligated DNA were purified by MinElute® PCR purification kit following the manufacturer's protocol. Briefly, 5 volume of Buffer PB was mixed to 1 volume of PCR product and put into MinElute column and centrifuged at 15,000 for 1 minute. After that, MinElute column was washed by Buffer PE and DNA was eluted in 15µl Buffer EB.

2.7.2. QIAquick gel extraction kit

DNA was extracted from agarose gel slices using QIAquick gel extraction kit following the manufacturer's protocol. Briefly, 3 volumes of Buffer QG were added to a 1.5ml microcentrifuge tube containing 1 volume of gel and the tube was incubated in 56°C for 10 minutes. Then, 1 volume of isopropanol was added to gel-resolved Buffer QG and the solution was transferred to a QIAquick column and centrifuged at 15,000 for 1 minute. QIAquick column was washed with Buffer PE and the DNA was eluted in 50µl Buffer EB.

2.8. Sequence

2.8.1. Nucleotide sequence

Sequencing was carried out using BigDye Terminator v3.1 (Applied Biosystems) according to the manufacturer's instructions. BigDye reaction was carried out in ABgene 0.2ml strip tube (Thermo scientific, UK.) which contained 7.5µl nuclease-free water, 2.7µl 5x BigDye Buffer, 0.6µl primer, 0.2µl BigDye Terminator v3.1, and 1µl PCR products. BigDye reaction was performed under following conditions: 20 cycles of 30 seconds at 96°C, 20 seconds at 50°C, and 4 minutes at 60°C. Sequences were read at the University of Edinburgh GenePool Sequencing Service. Sequences, with vector nucleotides removed, were submitted for analysis using the

NCBI Basic Local Alignment Search Tool for nucleotide or translated sequence homology (blastn or blastx). Sequences were considered candidate viruses if the top hits of either BLASTn or BLASTx were viral in origin.

2.8.2. Protein coding sequence

Protein coding sequences in complete genomes were identified using either Simmonic Sequence Editor v1.6 software or NCBI ORF Finder. The sequence data from section 2.8.1 were submitted to these programmes and the predicted proteins were analysed using NCBI protein BLAST. Phylogenetic trees based on the virus complete sequences were constructed using MEGA (version 4.1).

Rodent TTV genomic map was constructed by using DNASTAR Lasergene 9 core Suite. Rodent TTV complete genomes were submitted and rodent TTV genomic maps were compared to the results of ORF finder which was mentioned above.

2.9. Molecular cloning

2.9.1. Ligation

For *Taq* polymerase (Promega) (section 2.5.1.) or AccuPrimeTM *Taq* High Fidelity PCR amplicons (Invitrogen) (section 2.5.2.), the pGEM®-T Easy vector kit (Promega) was used for ligation and transformation. For ligation reactions, 2.5µl 2x ligation buffer, 0.5µl pGEM®-T Easy vector, 0.5µl T4 DNA Ligase and 1.5µl PCR amplicon were mixed in a PCR tube and incubated at 16°C overnight or 4°C for 2 days.

2.9.2. Transformation of competent cells with plasmid DNA

The recombinant DNA was inserted into DH 5 α TM competent cells (Invitrogen). The ligation mixture was transferred into the tubes containing competent cell (150 μ l) and incubated on ice for 30 minutes. After this, the tube was put into the 42°C water bath for 30 seconds before cooling back in ice for 2 minutes. In the tube, 150 μ l LB broth was added to the competent cells-ligation reaction mixture. The cells were incubated at 37°C for 1 hour with horizontal shaking. The cells were spread on LB agar plates containing ampicillin (100 μ g/ml), IPTG (0.5 μ M), and X-gal (80 μ g/ml) and incubated at 37°C overnight.

2.9.3. Colony screening PCR

For colony screening, primers specific for the M13F (GTA AAA CGA CGG CCA G) and M13R (CAG GAA ACA GCT ATG AC) sites located on opposite sides of the multiple cloning site of the pCR-Blunt II-TOPO (section 2.10.3.) or pGEM T easy vector were used. Plasmid-containing colonies were picked from LB/Agar plates directly into PCR master mix. Reactions were performed under the following conditions: Initial bacterial lysis/denaturation 2 minutes at 94°C, 30 cycles of [18 seconds at 94°C, 30 seconds at 50°C and 90 seconds (3min when PCR products is 2-3kb) at 72°C] and a final extension of 6 minutes at 72°C.

2.9.4. Plasmid DNA extraction

2.9.4.1. Small scale isolation of plasmid DNA using a QIAprep Miniprep kit QIAGEN

Plasmid DNA was extracted from the competent cells using miniprep kit (QIAGEN, UK) according to the manufacturer's protocol. Briefly, a single isolated bacterial colony was incubated in 5ml sterile LB broth containing the required antibiotics (100 μ g/ml ampicilline) for 37°C overnight. The culture was centrifuged at full speed for 5 minutes and the supernatant was discarded. The pellet was resuspended in Buffer P1. After that, Buffer P2 was added and mixed

by inversion. Buffer N3 was then added to the mixture and mixed by inversion. The tubes were centrifuged for 10min and the supernatant was applied in QIAprep spin column. The column was centrifuged and washed by Buffer PB and Buffer PE. Finally, plasmid DNA was eluted into 20µl of buffer EB.

2.9.4.2. Large scale isolation of plasmid DNA using an Endo free maxiprep

Plasmid DNA was extracted from the competent cells using Endo free maxiprep (QIAGEN, UK) according to the manufacturer's protocol. Briefly, 250ml LB broth which contained a single colony was incubated in 37°C overnight. The culture was centrifuged at 6000 x g for 15 minutes at 4°C. The cell pellets were resuspended by Buffer P1 and Buffer P2 was added in the mixture and incubated in 5min. Buffer P3 was added and applied in QIAfilter cartridge and incubated in 10min at room temperature. The mixture was filtered and Buffer ER was added to the filtered mixture. Then, the mixture was incubated 30 min on ice. The mixture was applied to a QIAGEN-tip 500 and was washed with Buffer QC twice. The plasmid DNA was eluted by Buffer QN and the eluted DNA was precipitated by the isopropanol. The DNA pellet was washed by 70% ethanol and redissolved by 1ml Buffer TE.

2.9.5. T4 ligation by using NEB T4 ligase

T4 ligation was carried out in ABgene 0.2ml strip tube (Thermo scientific, UK.) which contained 8µl distilled water, 1µl T4 ligation buffer (NEB), 1µl T4 ligase (NEB) and 100ng DNA. The mixture was incubated in 16°C overnight.

2.10. Construction of DNA libraries

2.10.1. Gel extraction

Before making a library, DNA was purified by agarose gel extraction. First, 5000µg DNA sample was run on agarose gel with 1kb ladder (DNA electrophoresis). Then, the area from 500 to 3000bp in agarose gel which should contain virus DNA was taken. After that, the agarose gel was extracted from the gel slice using QIAquick gel extraction kit (section 2.7.2.) and eluted in 30µl.

2.10.2. Rolling Circle Amplification

Rolling circle amplification (RCA) was performed by using the Repli-G Mini Kit (Qiagen) following to manufacturer's instructions with the exception that Phil-29 polymerase (NEB) was utilized as enzyme instead of the supplied enzyme, and the denaturation step before amplification was omitted. About 10-100ng DNA from liver or spleen was used as a template for rolling cycling amplification. The reaction was performed in 0.6 ml tube containing 1µl NEB phil-29, 29µl reaction buffer and 10µl H₂O and the mixture was incubated at 30°C overnight.

2.10.3. Digestion by restriction enzyme and cloning and sequence

RCA amplicons were digested by BstUI restriction enzyme (NEB) at 50°C for 4 hours and terminal phosphates were removed by Antarctic phosphatase (NEB) following to the manufacturer's instructions. Then, DNA fragments were purified with QIAquick gel extraction kit (section 2.7.2.) and eluted in 30µl. These DNA fragments were ligated into the pCR-Blunt II-TOPO vector (Invitrogen) and was transformed into One shot ® Top 10F' chemically competent *E. coli*. Ligations were performed in ABgene 0.2ml strip tube (Thermo scientific, UK.) containing 2µg PCR product, 0.5µg salt solution and 0.5µg PCR® II-Blunt-TOPO®. Reactions were incubated at room temperature 5 minutes. The ligation mixture was transferred into tubes containing One shot ® Top 10F' chemically competent *E. coli* (150µl) (Invitrogen) and it was

incubated on ice for 30 minutes. After that, this tube was put into the 42°C water bath for 30 seconds before cooling back on ice for 2 minutes. Then, 250µl S.O.C broth (Invitrogen) was added into the competent cells-ligation reaction mixture. The cells were incubated 37°C at 1 hour with horizontally shaking. The cells were spread on LB agar plates containing kanamycin (50µg/ml).

2.11. Development of assay for gene expression analysis using quantitative real-time polymerase chain reaction (real-time-qPCR)

2.11.1. Generation of standard curve by using TTV complete genome plasmid.

To set up the standard curve, rodent TTV complete genome containing plasmids were serially diluted from the 1×10^7 down to 10^0 copies/µl. The number of copies of plasmid in a microliter was determined using the following formula: $1\mu\text{g of } 1000\text{bp DNA} = 1.52 \text{ pmol} = 9.1 \times 10^{11}$ molecules. In the RoTTV1 plasmid, $9.1 \times 1 / 5,298\text{bp}$ (TTV complete genome 2,283bp and pGEM-T easy vector 3,015bp) = 1.72×10^{11} TTV copies/µg. DNA concentration (ng/µl) was measured by using Nanodrop (section 2.2.2) and 1.72×10^{11} TTV copies/ µg x DNA concentration (ng/µl) (change to µg/µl) = TTV copies/µl. In the RoTTV2, $9.1 \times 1 / 5,564\text{kbp}$ (TTV complete genome 2,549bp and pGEM-T easy vector 3,015bp) = 1.64×10^{11} TTV copies/µg. DNA concentration (ng/µl) was measured by using Nanodrop (section 2.2.2) and 1.64×10^{11} TTV copies/ µg x DNA concentration (ng/µl) (change to µg/µl) = TTV copies/µl.

2.11.2. Components of Real Time PCR reactions

Real time PCR analysis was performed using a Rotorgene (Corbett Research, Australia) and a hydrolysis probe based detection solution (Taqman®) for q-PCR and q-RTPCR. Reactions were carried out in a 20µl final volume which contained 8µl water, 10µl Brilliant III ultra Fast QPCR Master Mix (Agilent Technologies, USA) and 50pmol of each primer and probes shown in Table

2.11.2. (Primer design, UK). Each reaction contained 1µl of extracted DNA as a template to measure the copy number. Negative control was ready in parallel with dH₂O used in place of template DNA. Standard dilution reactions were performed with plasmid DNA by diluting in 50ng/µl salmon sperm DNA (Fisher Scientific UK Ltd, UK). Reactions were carried out in 0.1ml tubes (Corbett Research, Australia). Following a hot start at 95°C for 10 minutes to activate the hot start Taq polymerase, the samples were amplified for 50 cycles. The PCR programme was a two-step qPCR programme and the cycles consisted of 95°C for 15 seconds (denaturation) and 60°C for 60 seconds (Data collection). All samples including the standard dilutions and negative controls were run in duplicate to assess accuracy during reaction set-up.

Target	Orientation	Length	Sequence
RoTTV1 primer (used in 5.3.)			
RoTTV1	Sense	24	5' CGY ACT AGA AGA ACT AGA AAA TGG 3'
RoTTV1	Antisense	23	5' CCT CTG ATA ACA CAT CTC CTA GT 3'
RoTTV2 primer (used in 5.3.)			
RoTTV2	Sense	19	5' ACC AGM AGC TCC AGA CAC A 3'
RoTTV2	Antisense	20	5' GTA GTC CTC CCC TTG CAG TA 3'
Probes: target, probe name, length, sequence			
RoTTV1	RoTTV1a	27	5' TCT GAC CTT GAG GTT CCT TCT CCC GCG 3'
RoTTV2	RoTTV2	32	5' ACA TCA GAG ACC CAA CAS AAA TCA ATC ACG AG 3'

Table 2.11.2. The primer pairs and probes used for the real-time PCR.

2.12. Rapid amplification of 5' and 3' cDNA ends (RLM-RACE)

RACE was performed using the GeneRacer™ Kit (invitrogen) according to manufacturer's instructions. Briefly, the 5' end of wild mouse RNA or cell culture RNAs was ligated to the GeneRacer™ RNA Oligo. The GeneRacer oligo gives a known priming site for the PCR, and

this GeneRacer oligo attached mRNA is transcribed into cDNA by using random primers and superscript III following the protocol in section 2.4. Finally, the cDNA was amplified by using GeneRacerTM 5' primer and reverse gene specific primer shown in Table 2.12.1 and Table 2.12.2. In the 3' Race, mRNA was reverse transcribed using the GeneRacerTM Oligo dT Primer to create the 3' priming site for the PCR. Subsequently, PCR was carried out using GeneRacerTM 3' Primer and forward gene specific primer shown in Table 2.12.1 and Table 2.12.2.

PCR was performed by using AccuPrimeTM *Taq* High Fidelity (Invitrogen) (section 2.5.2.). Nested touchdown PCR was chosen to remove the non-specific PCR products. Touchdown PCR programme was set up under the following condition: 5 cycles of 5 seconds at 94 °C, 3 minutes at 72°C, then, 5 cycles of 5 seconds at 94 °C, 3 minutes at 72°C, and finally 25 cycles of 5 cycles of 5 seconds at 94 °C for denaturation, 10 seconds at 68 °C and 3 minutes at 72 °C. This first round PCR product was diluted 5µl in 245µl with distilled water and 5µl diluted first PCR products were used as the template for second round touchdown PCR and the programme was changed to 20 cycles in final step of touch-down PCR. The amplified PCR was cloned and sequenced using pGEM®-T Easy vector kit (Promega) (section 2.9.1).

Target	Orientation	Length	Sequence
GeneRacer™ Primer			
GeneRacer™ 5' primer	Antisense Outer	23	5' CGA CTG GAG CAC GAG GAC ACT GA3'
GeneRacer™ Nested 5' primer	Antisense inner	26	5' GGA CAC TGA CAT GGA CTG AAG GAG TA 3'
GeneRacer™ 3' primer	Sense Outer	25	5' GCT GTC AAC GAT ACG CTA CGT AAC G 3'
GeneRacer™ Nested 3' primer	Sense Inner	23	5' CGC TAC GTA ACG GCA TGA CAG TG 3'
3' Race Gene specific primer			
ORF2 Gt1a OS	Sense Outer	27	5'CCT GGA CCA CAC ACT TGA AGA CCC TAC 3'
ORF2 Gt1a IS	Sense Inner	30	5' GGC GTG GAG ATT CCC AAG ACA GAG ARC C3'
O3 Race Out	Sense Outer	31	5' GGT ACA CGA CAA GCA AGA ACA CCG ACA CCA G 3'
O3 Race In	Sense Inner	32	5' CAC CGA CAC CAG CTC GAC AGA ATC ATC ATC AG 3'
Whole transcript primer			
ORF2 OS	Sense Outer	30	5' GAT TTT ATC TAC AAG GAG GAT TCA TGG CTG 3'
ORF2 IS	Sense Inner	27	5' GGC TGT GCA TGA TCA GGC AAA CTC ATG 3'
ORF3 OAS	Antisense Outer	30	5' GAT AAC TGA GGA TCC ACA GGC ACA TTG GAG 3'
ORF3 IAS	Antisense Inner	30	5' CAT ATT TTG AGA AGG TGT CAG TGG CGG CCC 3'
5' Race Gene specific primer			
5 O2 OUT	Antisense Outer	29	5' GTT CTT CTA GCA CGC CGA GCA GTT CTT CG 3'
5 O2 IN	Antisense Inner	29	5' GTA GGT GCG CCG TCT GGT TGG TCT CCA GG 3'

Table 2.12.1. GeneRacer primer and race gene specific primer to detect the transcript of RoTTV1

Target	Orientation	Length	Sequence
3' Race Gene specific primer			
Gt2 3Race O	Sense Outer	29	5' CCG ACT CTT CTG TGA CTG CGG GGA CCC TG 3'
Gt2 3Race I	Sense Inner	30	5' GTA CTT CTA CCG GCG GAC CTT TCG TTA CCC 3'
Whole transcript primer			
Gt2 O4 end OAS	Antisense Outer	27	5' CTC ACC AAC TAA TTT CAA TCC CAC ATC 3'
Gt2 O4 end IAS	Antisense Inner	27	5' CAA ACG GGC GGT GGA TGG GTG ACA CGG 3'
5' Race Gene specific primer			
Gt2 5Race O	Antisense Outer	29	5' GTT CTT CTA GCA CGC CGA GCA GTT CTT CG 3'
Gt2 5Race I	Antisense Inner	29	5' CAG GGT CCC CGC AGT CAC AGA AGA GTC GG 3'

Table 2.12.2. Race gene specific primer to detect the transcript of RoTTV2

2.13. Protein expression of rodent TTV transcripts

2.13.1. Primer design of RoTTV transcripts amplification

For the protein expression, pEGFP-C1 (Clontech) and pcDNATM 3.1/ myc-His (-) A MCS (Invitrogen) were used for the protein expression. To combine the protein predicted coding sequence to the plasmids, the coding sequences were amplified by using the primer pairs which contained the restriction sites. The primers used for this study were shown in Table 2.13.1. PCR programme was same as the standard PCR shown in 2.5.1. Bone marrow cDNA from an infected mouse was used as the template for the spliced transcripts and TTV complete genome was used as the template for un-spliced transcripts. For the spliced transcripts, nested PCR was carried out by using the first round PCR primer TC-1st PCR (Table 2.13.1.1.).

Target	Orientation	Length	Sequence
*1 st round PCR to amplify the spliced transcripts			
TC-1st PCR; F	Sense	34	5' CAG GAT GAA TGT GGA TGT GGA TGA AGA TTT TAT C 3'
TC-1st PCR; R	Antisense	23	5' CAG AAT TCA ATG TTT ATT GGG GG 3'
†pEGFP-C1 restriction site containing primer 5' GG(CC)---restriction site--primer 3'			
pEGFP-C1 ORF1:F Restriction site; HindIII	Sense	34	5' GG---A AGC TT--- C CAT GGC GTG GAG ATT CCC AAG ACA GAG 3'
pEGFP-C1 ORF1:R Restriction site; Xba I	Antisense	34	5' CC---T CTA GA--- G GTG ACA GGA ACA TCC ATG TCT GG 3'
pEGFP-C1 ORF1-4:R Restriction site; Xba I	Sense	29	5' CC---T CTA GA--- A ATA CAG ACG ACT CTG TTG AG 3'
pEGFP-C1 ORF2 :F Restriction site; HindIII	Sense	34	5' GG---A AGC TT--- C CAT GAA TGT GGA TGA AGA TTT TAT C 3'
pEGFP-C1 ORF2:R Restriction site; Xba I	Antisense	29	5' CC---T CTA GA--- T GAG AGG ACA CAT AAT GCC TA 3'
pEGFP-C1 ORF2-3:R Restriction site; Xba I	Antisense	31	5' CC---T CTA GA--- A TGT TTA TTG GGG GAT AAC TGA G 3'
†pcDNA TM 3.1/ myc-His(-) A MCS restriction site containing primer 5' GGG(CCC)---restriction site--primer 3'			
pcDNA ORF1:F Restriction site; Xba I	Sense	35	5' GGG ---CT AGA--- ATG GCG TGG AGA TTC CCA AGA CAG AG 3'
pEGFP-C1 ORF1:R Restriction site; HindIII	Antisense	32	5' CC---A AGC TT--- G GTG ACA GGA ACA TCC ATG TCT GG 3'
pEGFP-C1 ORF1-4:R Restriction site; HindIII	Antisense	29	5' CC---A AGC TT--- A ATA CAG ACG ACT CTG TTG AG 3'
pEGFP-C1 ORF2:F Restriction site; Xba I	Sense	33	5' GGG---TCT AGA--- ATG AAT GTG GAT GAA GAT TTT ATC 3'
pEGFP-C1 ORF2:R Restriction site; HindIII	Antisense	29	5' CC---A AGC TT--- T GAG AGG ACA CAT AAT GCC TC 3'
pEGFP-C1 ORF2-3:R Restriction site; HindIII	Antisense	31	5' CC---A AGC TT--- A TGT TTA TTA TTG GGG GAT AAC TGA G 3'

Table 2.13.1. The primers which were used to construct the transcripts containing pEGFP-C1 and pcDNATM 3.1/ myc-His(-) A MCS. *For the spliced transcript, 1st PCR primers were used for the amplification for the first round PCR. †The primers which have the restriction site to inset to the plasmid. †They also have extra G or C to avoid the mispriming of restriction site.

2.13.2. Transfection of pEGFP-C1 and pcDNATM 3.1/ myc-His(-) A MCS constructs

The plasmids which contain the coding regions identified from the transcripts were transfected by using lipofectamineTM 2000 reagent (Invitrogen, UK) following the manufacturer's protocol. Briefly, 5×10^4 of BHK cells (section 2.15.1.1.) were seeded into 24 well plates and incubated one day before the transfection. Two μg of plasmid DNA was diluted in serum/antibiotic free growth medium to make the total volume 50 μl and the 1 μl of lipofectamine 2000 was diluted in serum/antibiotics free growth medium to make the total volume 50 μl . Diluted lipofectamine was incubated in 5 minutes at the room temperature and mixed with the diluted plasmid, and the mixtures were incubated 20 minutes at room temperature. The mixture was added to the well in 24 well plates which contained 150 μl growth medium and cells and incubated 5 hours at 37°C in humidified 5% CO₂. Because of the toxicity of lipofectamine 2000, if the cells were fragile (such as the stem cell culture), the medium had to be replaced with the fresh medium after 5 hours and the cells were incubated 24-48 hours.

2.13.3. Harvesting for western blot

Adherent cells were removed from the tissue-culture flask by pipette tip to harvest the cells. Firstly, the growth medium was taken from the well and the proteinase inhibitor (cOmplete ULTRA Tablet, Roche) dissolved in PBS was put into the well. The bottom of the flask was scratched by the pipette tip and the removed cells were kept in 1.5ml microcentrifuge tubes (Eppendorf, UK). Samples were centrifuged for 5 minutes for 450 x g. The supernatant was discarded and one volume of 2x sample buffer Laemmli (Sigma-Aldrich) was added to the sample and boiled for 5 minutes. The samples were used for western blot (section 2.14.).

2.13.4. Staining of Transfected Cells

For the staining, 2×10^4 cell of BHK-21 cells (section 2.15.1.1.) were seeded into BC Falcon Culture slide 8 well (Scientific Laboratory Supplies Ltd., U.K.) and were incubated at 37°C with 5% CO₂ for 24-48 hours to 70% confluence. 0.8 µg of pEGFP-C1 and pcDNATM 3.1/ myc-His(-) A plasmid constructs were transfected into these BHK-21 cells (section 2.13.2.) and incubated at 37°C with 5% CO₂ for 24-48 hours until 85-90% confluent.

For the pcDNATM 3.1/ myc-His(-) A, transfected cells required immunostaining for recombinant protein detection. The wells of culture slide were removed following the manufacturer's protocol. The cells were fixed for 10 minutes in 4% (w/v) paraformaldehyde and washed twice for 5 minutes with PBS. The cells were permeabilised by incubation with PBS containing 0.25% (v/v) Triton X-100 (*t*-Octylphenoxypolyethoxyethanol, Sigma) for 20 minutes followed by two 5 minutes washes in PBS.

Slides were blocked with normal rat serum (Sigma) diluted 1:10 in CAS-BlockTM (Invitrogen) for 30 minutes. Blocking solution was poured off and the cells were stained with Anti-c-myc

antibody (9E10 from mouse ascites fluid, Invitrogen) diluted 1 in 300 in CAS-Block™ (Invitrogen) for 1 hour. The primary antibody was detected using FITC polyclonal anti mouse IgG (Pharmingen) diluted 1 in 100 for 30 minutes in room temperature in the dark. The secondary antibody was poured off and the slides were washed three times in PBS for 5 minutes. DAPI (diluted 1:1000 by PBS, invitrogen) or TO-PRO-3 iodide (Molecular Probes, diluted 1:1000 to 1µM by PBS) were put on the washed slide and incubate 30 minutes for the counterstain. Then, the slides were washed three times in PBS for 5 minutes each. Coverslips were mounted using MOUNT PERMALUOR (Thermo) and stored, wrapped in foil, at 4°C.

The cells transfected with GFP constructs did not require antibody staining and the slides were fixed, permeabilised and counterstained by TO-PRO-3 iodide as described above.

2.13.5. Apoptosis staining by FLICA apoptosis detection kit

For the understanding of the apoptosis inducing capacity of virus protein, apoptosis staining was performed. Apoptosis was detected by FLICA® 660 *in vitro* Poly Caspase Detection kit (Immunochemistry Technology LLC) following the manufacturer's protocol. The GFP transfected cells were fixed and washed as described in section 2.13.4. FLICA stock solution was diluted in 1:50 and put on the slides and incubated for 60 minutes. The stained slides were washed with PBS for 5 minutes. Again the slides were washed 1x Apoptosis Wash Buffer. DAPI (1:1000 by PBS) was used for the counterstain. Coverslips were mounted using MOUNT PERMALUOR (Thermo) and stored, wrapped in foil, at 4°C.

2.13.6. FACS Analysis

FLICA stained and the fusion protein expressed cells were also analysed by FACS machine. Forward scatter (cell size), side scatter (cell granularity), GFP fluorescence (B530/30), and

FLICA fluorescence (R670/14) of the cells were measured on a LSR Fortessa Flow cytometer (BD Biosciences). Data were analysed using both Cell Quest (BD Biosciences) and WINMDI (<http://facs.scripps.edu/software.html>) software.

2.13.7. Confocal microscopy

Slides of transfected cells were viewed using a Zeiss LSM710 confocal microscopy (Carl Zeiss, UK) at the Roslin institute, University of Edinburgh with the assistance of Mr. Bob Fleming.

Image analysis was performed using ZEN blue 2011 (Carl Zeiss, UK).

2.14. Western blot

2.14.1. Protein Electrophoresis

The cells from the cell cultures were added to the one volume of 2x SDS sample buffer Laemmli (Sigma-Aldrich) in 1.5ml microcentrifuge tubes (Eppendorf, UK) (section 2.13.3.) and heated at 100°C for 5 minutes. Then, the tubes were centrifuged to precipitate the insoluble pellet. The glass gel plates were assembled with spacers using Mini-PROTEAN Tetra cells (Bio-Rad). Resolving gels containing 15% acrylamide was poured just after the addition of ammonium persulphate (APS) and N, N, N', N'-Tetramethylethylenediamine (TEMED) (see below). The gel was poured to just below the comb inserting area and overlaid with sec-butanol to prevent drying. Resolving gel was polymerised for 30 minutes and stacking gel was prepared using this time without APS (see below). Then, the sec-butanol was removed and resolving gel was washed twice by the distilled water. The APS was added to the stacking gel which was then poured and the comb was inserted. The stacking gel was polymerised for 30 minutes. The comb was removed and the gels were assembled in the Mini-Protean II gel tank. The SDS running buffer (see below) was poured in the assembled gel holder firstly and the remaining buffer was poured in the reservoir. The boiled samples and PageRuler Prestained protein Ladder (Thermo) were

loaded into each well and run at 30 mA for 1 hours until the samples and ladder had reached the bottom of the gel.

SDS recipes

4%Stacking Gel

H₂O; 2.47ml

Acrylamide/bis-Acrylamide (37.5:1); 0.53 ml

Stacking Gel Buffer (0.5M Tris-HCL, 0.4% SDS pH 6.8); 1.00ml

N, N, N', N'-Tetramethylethylenediamine (TEMED); 1.4µl

Ammonium Persulphate (APS); 90µl

Resolving Gel (10%)

H₂O; 2.92ml

Acrylamide/bis-Acrylamide (37.5:1); 2.33ml

Resolving Gel Buffer (1.5M Tris-base, 0.4% SDS pH8.9)

N, N, N', N'-Tetramethylethylenediamine (TEMED); 2.8µl

Ammonium Persulphate (APS); 90µl

SDS Running Buffer

Tris; 5.46g

Glycine; 25.68g

Sodium Dodecyl Sulphate; 1.9g

Make up to 900µl

2.14.2. Transfer of Protein to Nitrocellulose Membrane

Transfer membrane, Immobilon ®-P (Millipore) was cut to the same size as the resolving gel (approximately 4x8 cm) and 4 pieces of Blotting paper (GE healthcare Life Science) were cut slightly larger than the transfer membrane. The membrane was soaked in methanol, and the blotting papers and two sponges were soaked in the transfer buffer (10 times diluted x10 premixed blotting buffers (Bio-rad) containing 20% methanol). For the wet transfer, Bio-rad Mini Trans-Blot Cell was assembled. Transfer unit was assembled in the gel holder cassettes following order; from cathode (-), one sponge, two blotting paper, resolving gel, membrane, two blotting paper and one sponge to anode (+) and the trapped air bobbles were removed by gently rolling the side of a pipette over the top sheet in each layer. The transfer unit was put in a modular electrode assembly and set on the buffer tank with Bio-ice cooling unit which contained ice to make the buffer cold. Finally, the transfer buffer was poured in the tank to cover the transfer unit and the lid was put on the tank. A current of 100V was applied for 1 hour. Following the transfer, the membrane was carefully taken and marked on the gel attached side. The membrane was stained with AMRESCO stain Ponceau S solution (BioExpress) for 1 minute and rinsed with PBS until the well-defined bands were appeared on the membrane to show the protein transfer.

2.14. 3. Immunological Detection of Protein Blot

For GFP and 6x His detection, protein blots were blocked in 5% skim milk in PBSt (0.05% (v/v) tween 20) for 1 hour. The primary antibody reaction was carried out by using anti-GFP (sc-8334, santa cruz) (1:500) or anti-his (C-term) HRP antibody (3D5, Invitrogen) (1:10000) diluted in 5% skim milk PBSt. The reaction was incubated overnight at 4°C. The following day, the membrane was washed three times in PBSt for 10 minutes each. Only for anti-GFP antibody, secondary antibody was required and Donkey anti-rabbit HRP antibody (AP182P, Chemicon (Millipore)) was used at 1:3000 dilution. The reaction was incubated for 30 minutes at 4°C. The membrane

was washed three times in PBSt for 15 minutes each. Bound antibody was detected by ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences) and the detection reagent 1 and 2 were mixed and applied on the membrane and the membrane was incubated for 5 minutes. The fluorescence was detected by Amersham Hyperfilm ECL in dark room and the film was developed by Medical Film Processor (SRX-101 A, Konica Minolta Medical & Graphic INL)

2.15. *In vitro* experiment

2.15.1. Maintenance of cell line cell culture

2.15.1.1. Baby hamster kidney (BHK-21) cell cultures

Baby hamster kidney (BHK-21) cells are a continuously growing fibroblastoid cell line derived from a Syrian golden hamster. BHK-21 cell were cultured in Glasgow modified eagle's medium (GMEM, Invitrogen, UK) supplemented with 10% (v/v) tryptose phosphate broth (TPB, Invitrogen, UK), 100U/ml penicillin, 100U/ml streptomycin (Merck BDH), 10% NBCS (newborn calf serum, Invitrogen) and 2mM L-glutamine (Merck BDH). Cell lines were cultured in sterile plastic-ware (Nunc) and incubated at 37°C in equilibrium with humidified 5% CO₂.

2.15.1.2. Mouse embryo fibroblast (MEF)

Mouse embryo fibroblast (MEF) alpha/betaSV1 line was used in infectious experiment. MEF cells were cultured in Dulbecco's modified Eagles's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen), 1% penicillin and streptomycin (penicillin 10,000U/ml and streptomycin 10,000U/ml) (Gibco, UK). Cells were incubated at 37°C in a 5% CO₂ incubator.

2.15.1.3. Passage

For further passaging, adherent cells were removed from the bottom of sterile plastic (Thermo Scientific Nunc™) by trypsinisation. Growth medium was removed and the cells were washed by using PBS. Incubation with 0.25% trypsin 1x Phenol red (Invitrogen) was used to detach cells from the plastic (Thermo Scientific Nunc™). An equal volume of growth medium was added to inactivate the trypsin. After that, the cells were centrifuged for 450 x g for 5 minutes. The supernatant was discarded and cells were resuspended in appropriate volume of fresh medium. In the 1.5ml tube, the resuspended cells were diluted 1 to 10 with the 0.1% trypan blue (w/v) and the number of unstained viable cells was counted using haemocytometer. Approximately 5×10^6 - 1×10^7 cells were re-seeded into fresh tissue culture flask in about 50 ml of medium.

2.15.2. Primary cell culture

2.15.2.1. Bone marrow derived macrophage

The bone derived macrophages were set up by using L929 medium (see below) and Revolutions-per-Minute Indicator (RPMI) medium (Invitrogen) containing 10% fetal calf serum (FCS, Invitrogen), 1% penicillin and streptomycin (penicillin 10,000U /ml and streptomycin 10,000U/ml) (Gibco, UK). Firstly, femurs were taken from the mouse intactly and placed in cold RPMI (Invitrogen). Secondly, all skin and muscles were removed and the femurs were dipped in 70% alcohol for 5 minutes to sterilise. The joint ends of femurs were cut with scalpel. Thirdly, a hypodermic needle (approx. 26 gauge) (Terumo ® Hypodermic needle) was inserted into femur and used to flush the bone marrow cells through femur into Falcon tube (BD Bioscience cell culture) by using RPMI in 10 ml syringe (Terumo). The femurs were flushed from both ends to harvest maximally. The other leg was also treated using the same way and the cells were collected in same Falcon tube. Finally, 20 ml L929 conditioned medium (the source of M-CSF, see below) were pooled with the cells in the Falcon tube and the medium containing the cells

were mixed gently and seeded into two square culture dishes (Thermo scientific Nunc™) and the plates were incubated at 37°C in equilibrium with humidified 5% CO₂. The cells were split on day 4 (50% each RPMI and L929 medium). For the further passage, day 4 growth medium was taken to Falcon tube. In the other Falcon tube, 1x DPBS (Invitrogen) was prepared and 10 ml of 1x DPBS was added to each plate. 10 ml of DPBS were taken in a 10 ml syringe (Thermo) with the medium gauge needle (approx.15 gauge) and the cells were washed off on the surface by vigorous pressure until washing all over the surface at all orientation of the plates. The collected cells in DPBS were put into the conical skirted bottom universal sample tube. The sample collection was repeated by using another 5ml 1x DPBS. The collected cells were pelleted by centrifugation at 15,000 xg for 5 minutes. The square culture dishes (Thermo scientific Nunc™) were reused for the next passage and 5 ml of old medium was added back to the old square culture dishes and new square culture dish respectively. The centrifuged cells were resuspended in 20 ml of 50% L929 and 50%RPMI and the cells in divided into the 2 square culture dishes (old and new). Macrophages matured and the cells used for the experiment at day 7.

L929 medium

L929 is a murine aneuploid fibrosarcoma cell line and known to secrete a beta-NGF-like molecule to develop the conditioned medium (Pantazis et al., 1983). L929cells were grown up in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen), 1% penicillin and streptomycin (penicillin 10,000U /ml and streptomycin 10,000U/ ml) (Gibco, UK) for 4-7 days at 37°C in equilibrium with humidified 5% CO₂. The growth medium contains factors which induce the monocytes to differentiate to macrophages.

2.15.2.2. Bone marrow derived stem cell

Bone marrow derived stem cells were also set up by using stem cell media (DMEM (section

2.15.1.2.) with additional recombinant proteins). Bone marrow cells were collected by using the same method as bone marrow derived macrophage (section 2.15.2.1.). For the stem cells, the cells were flushed by stem cell media (DMEM, 10% FCS, 5ml of Glutamax, 5ml of Penicillin/streptomycin + (stem cell factor) SCF 100ng/ml, IL-3 10ng/ml, IL-6 50ng/ml). After the collection, the other 20 ml of stem cell media were added to the cells and incubated in square culture dishes (Thermo scientific NuncTM) at 37°C in equilibrium with humidified 5% CO₂. The following day, many dead cells are found, and they were cleaned up by centrifugation and resuspension of the cells in fresh stem cell media. The cell density should be 10⁶ cells/ml and the cells matured in day 2-3 for use in subsequent experiments.

2.15.3. Transfection

For the cell lines transfection, Lipofectamine 2000 (Invitrogen) was used and the protocol was shown in section 2.13.2. Stem cells were very fragile and lipofectamine 2000 was seen to damage the cells. Accordingly, SAFectinTM-STEM transfection reagent (Deliverics) was used for the stem cell transfection following the manufacturer's protocol. Briefly, mouse stem cells were seeded into 24 well plate and incubated one day before the transfection to make 60-80% confluence. Two 0.6 ml microcentrifuge tubes (Sigma-Aldrich) containing 50µl of serum-free medium were prepared, and the appropriate volume of DNA and SAFectinTM-STEM were added to each tube respectively and the tubes were vortexed for 5 seconds. These two tubes were mixed together and incubated at room temperature for 20 minutes. The incubated mixture was added to each well containing the stem cells and 400µl growth media and mixed gently by rocking the plate back and forth. The transfected cells were incubated at 37°C in equilibrium with humidified 5% CO₂.

2.15.4. Primers for construction of plasmid for the transfection

2.15.4.1. Single copy of RoTTV complete circular genome.

Primers were designed based on the RoTTV complete genome. The restriction site was found in RoTTV complete genome and the inverted primers were constructed to contain the restriction site on RoTTV genomes (Spe I for RoTTV1 and HindIII for RoTTV2) (Table 2.15.4.1.). PCR condition was same as described in section 2.5.1. The details of single copy of RoTTV complete circular genome construction was shown in section 5.2.3.

Target	Orientation	Length	Sequence
RoTTV1 (Spe I site)			
1012 Gt1a sp SpeI F	Sense	23	5'ACT AGT AAC AAT TCT GAT CAG TA 3'
1012 Gt1a sp SpeI R	Antisense	19	5' ACT AGT CTG CAA CAT CTG C 3'
RoTTV2 (Hind III site)			
Gt2H HindIII F	Sense	28	5' AAG CTT ATC AAC AAA AAA ATT ATG GGAT 3'
Gt2H HindIII R	Antisense	24	5' AAG CTT TAT TTT TTT TGT TAG GCT 3'

Table 2.15.4.1. The primers containing the restriction site on RoTTV genome

2.15.4.2. Construction of GFP/ RoTTV1

GFP/RoTTV1 was constructed by the amplification of complete GFP coding sequences and RoTTV1 genomes with novel restriction sites added during PCR using the restriction site containing GFP and RoTTV1 primers (Table 2.15.4.2.). PCR conditions were the same as described section 2.5.1. The detail of GFP/RoTTV-1 construction was shown in section 5.2.5.

Target	Orientation	Length	Sequence
* RoTTV1 with the restriction site 5' GG(CC)---restriction site--primer 3'			
RoTTV F+Hind III Restriction site; HindIII	Sense	22	5' GG---A AGC TT---- ATG GCG TGG AGA TT 3'
RoTTVR+BamHI Restriction site; BamHI	Antisense	25	5' CC---GGATCC---TCC GGA TGG TTA AAG TC 3'
*GFP amplification with the restriction site 5' GG(CC)---restriction site--primer 3'			
GFP F + Bam HI Restriction site; BamHI	Sense	27	5' GG ---GGATCC---ATG GTG AGC AAG GGC GAG G 3'
GFP R + Hind III Restriction site; HindIII	Antisense	25	5' GG ---A AGC TT---CTT GTA CAG CTC GTC CGT CCA 3'

Table 2.15.4.2. The primers with the restriction sites for GFP/TTV construction.

2.15.4.3. Myc tagged concatemerised RoTTV

Myc tagged RoTTV1 complete genome was constructed by amplification of the complete genome by PCR using a primer encoding the c-myc tag (Table 2.15.4.3.). PCR condition was the same as described section 2.5.1. The detail of myc tagged concatemerised DNA construction was shown in section 5.2.6.

Target	Orientation	Length	Sequence
* Myc RoTTV1 F: 5' GGG(CCC)---restriction site--primer 3' R: 5' GGG(CCC)---restriction site--Myc--primer 3'			
Myc TTV Gt1a F Restriction site; Xba I	Sense	33	5' GGG---TCTAGA--- ATT TTG ACA ATC CAG TGG TGAGGC3'
Myc TTV Gt1a R Restriction site; Xba I	Antisense	69	5' CCC---TCTAGA---CAG ATC CTC TTCTGAGAT GAG TTT TTG TTC---CTG TTT ATT GGG GGA TAA CTG AGG ATC3'

Table 2.15.4.3. Myc tagged RoTTV1 complete genome primer. Myc tag was attached on the reverse primer.

2.15.4.4. RoTTV1 splicing site primer

To detect the transcripts only, the primers were designed spanning the splicing site of RoTTV1. The primers were shown in Table 2.15.4.4. By using the primers, the amplified products of 1080 bp in size would be amplified from RoTTV1 genomic DNA and 180 bp in size would be shown from RoTTV1 spliced cDNA. PCR condition was the same as described in section 2.5.1.

Target	Orientation	Length	Sequence
RoTTV1 splicing site primer (used in 5.7)			
1012 sp OAS	Antisense Outer	25	5' GGA CAC ATA ATG CCT CTG ATA ACA C 3'
1012 sp IAS	Antisense Inner	24	5' GTG TAT CTA GGC TGT ATT CCA GTG 3'
1012 sp OS	Sense Outer	28	5' CAA GAC AGA GAA CCT GGA GAC GGA AAA C3'
1012 sp IS	Sense Inner	23	5' GGA GAC CAA CCA GAC GGC GCA CC 3'

Table 2.15.4.4.. RoTTV1 splicing site primer.

2.16. *In vivo* transfection

In the 1.5 ml Eppendorf Tube (Eppendorf), 1µg (in 2µl) of RoTTV DNA and 57.7 µl of 150 mM NaCl were mixed for *in vivo* transfection. 0.3 µl of 0.1 M polycation polyethylenimine (**PEI**) (see below) was added dropwise and vortexed intermittently. The mixture was incubated at room temperature for 15-20 minutes before inoculating the mice. The 60 µl of PEI and RoTTV DNA complex was inoculated into the mice intranasally.

Stock 0.1 M PEI (4.3 mg/ml in PBS (x1))

PEI was measured by using the glass beaker and dissolved in the PBS. The stock PEI was adjusted to pH 7.4 with HCL. The reagent was filtered through 0.2µm filter (Millipore).

CHAPTER III. CHARACTERISATION OF RODENT TORQUE TENO VIRUS IN WILD RODENT POPULATIONS

TTV genetic diversity and high infectious distribution have been reported in animal and human populations. RoTTV (RoTTV) infectious prevalence, host species distribution, and complete genome sequences were revealed in this study. A phylogenetic tree was constructed and RoTTV was compared to human and other animal TTV.

3.1. Background to the study

3.2. Infectious prevalence of RoTTV in wild rodents using pan-TTV primers

3.3. Sequence results of screening PCR amplicons

3.4. Amplification of RoTTV full-length genomes

3.5. Complete sequence and phylogenetic analysis of RoTTV

3.6. Genomic structure and coding capacity

3.7. Design of species specific primer sets for screening RoTTV genomic prevalence in wild rodent

3.8. Discussion

Appendix 1. Rat TTV infection in lab rats

3.1. Background to the study

Infection with anelloviruses is not restricted to humans. By using highly conserved primers and RCA, TTV-related viruses were discovered in farm animals (Leary *et al.*, 1999), pets (Okamoto *et al.*, 2002), and wild animals (Okamoto, 2009). These TT viruses were species-specific, and the genetic sequence and genome size were quite variable (Hino *et al.*, 2007). TTV was found in dogs and cats, and TTV found in feline is the smallest in size (2064nt) (Okamoto *et al.*, 2002).

TTV research has several difficulties (section 1.7.) and a suitable animal model is required. Currently, porcine TTV is the first candidate because the epidemiology of porcine TTV and the porcine immune system resemble those of humans (Kekarainen and Segalés, 2007). Thus, porcine TTV is considered a good animal model for TTV infection. Also, non-human primate TTV has also been studied for the pathogenesis research because non-human primate TTV resembles human TTV (Tawara *et al.*, 2000).

The studies for animal TTV would be useful because these human and animal TTV have several common features. Firstly, animal and human TTV show a high level of genetic heterogeneity. In human TTV, at least 39 genotypes with a difference of greater than 30% and five major genetic groups with a difference greater than 50% have been found (Hallett *et al.*, 2000). On the other hand, chimpanzee has three TTV genotypes including a TTMV-like type (Okamoto *et al.*, 2000; Inami *et al.*, 2000) and pig has three TTV genotypes (Okamoto *et al.*, 2002; Niel *et al.*, 2005). Smaller TTVs were also observed in the several animals (section 1.1.4.). Secondly, TTV genomic structure is relatively conserved among the different TTV genotypes. TTV, TTMDV and TTMV in human and animals have a common presumed four open reading frames (ORFs, ORF1-ORF4) (Okamoto *et al.*, 2009). Third, co-infection with different genotypes of TTV, TTMDV and/or

TTMV in same individual is common in human and animal TTV infection (Maggie and Bendinelli., 2010).

The mouse is one of the most useful animal models in all biological research and many papers have been published using mouse models (Steven *et al.*, 2012; Liu *et al.*, 2013; Hajj *et al.*, 2012; Lemmermann *et al.*, 2012; Niels *et al.*, 2012). Mouse animal models have many advantages. The various knockout mice give us the hints to understand the immune response to virus infection. Transgenic mice provide the insight into the specific genes and cytokines involved in virus infection. Also, mice are small size which reduces the amount of research reagent or drugs and only small space is required to keep them. The high reproduction rate of mice provides the abundant samples. Inbred strains can remove the difference among the individuals and stabilize research results (Webre *et al.*, 2012).

RoTTV was discovered in the wood mouse by using a shotgun cloning technique combined with RCA. The UTR of this original clone was found to be compatible with existing pan-TTV primers so these could be used for a broader population study. We planned to use RoTTV as an animal model because mice are readily available, inexpensive to maintain, and a large number of reagents are available to study them. To start the study, we have to understand about the nature of RoTTV infection in wild rodent populations.

In this chapter, we will characterise RoTTV in the wild rodent and understand the epidemiology, natural host, genotypes, and genetic structure.

3.2. Infectious prevalence of RoTTV in wild rodents using pan-TTV primers

RoTTV was first discovered in the wood mouse (*Apodemus sylvaticus*). However, there is a possibility that RoTTV infects other rodent species in the wild. Therefore, a broader study of RoTTV infectious prevalence and genetic diversity was investigated in order to understand RoTTV. In the wild rodent population in United Kingdom, four wild rodents species were investigated; wood mice (*Apodemus sylvaticus*), field voles (*Microtus agrestis*), bank voles (*Myodes glareolus*), and house/lab mice (*Mus musculus*). These rodent species were classified by using RFLP (Restriction Fragment Length Polymorphism) on a PCR for mitochondrial cytochrome B and confirmed by sequencing of these amplicons. RFLP is the method to characterise the DNA (species in this study) by using a difference in homologous DNA sequence that can be shown by the presence of fragments of different lengths after digestion of the DNA samples with specific restriction enzyme. In this study, mitochondrial cytochrome B DNA was digested by RsaI, and house/lab mice DNA have one restriction site and field voles have several restriction site in mitochondrial cytochrome B DNA. Wood mice and bank voles have no restriction site on RsaI digestion. A gel image of restriction enzyme digested DNA is shown in Fig.3.2.1. House/lab mouse DNA produced two fragments (Lane 8-11) after RsaI digestion and field voles DNA were finely digested by RsaI (Lane; 12-13), but wood mice and bank voles DNA showed no digestion. Table 3.2.1. shows the rodent species which were used in this project. By using RFLP method, the wild samples were confirmed as house/lab mouse (*Mus musculus*,) n=24, field voles (*Microtus agrestis*) n=80, wood mouse (*Apodemus sylvaticus*) n=126 and bank voles (*Myodes glareolus*) n=39. Mice from pet shop and laboratory were confirmed as *Mus musculus*.

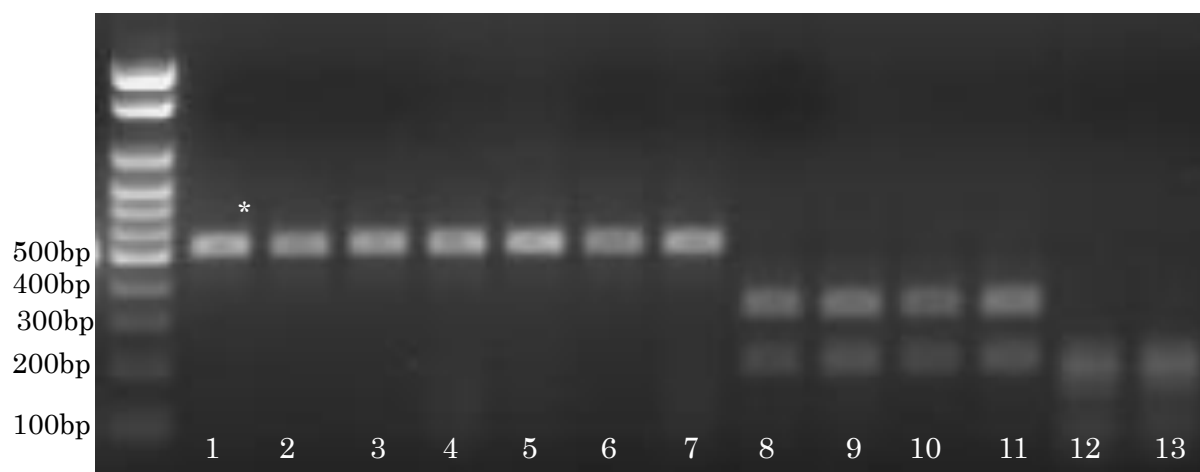


Figure 3.2.1. Restricted digestion of mitochondrial DNA PCR amplicons. PCR amplicons were digested using *RsaI*. Digested wood mouse and bank vole DNA showed single uncut band (550 bp)(band; 1-7)* but digested house/lab mouse DNA was cut and makes two DNA fragments, 200 bp (lower) and 350 bp (upper) (bands; 8-11). Digested field vole DNA was finely cut by *RsaI* (12-13).

Species	Wood mouse (wild)	Field vole (wild)	Bank vole (wild)	House mouse (wild)	House mouse (lab)	House mouse *(feeder)
number	126	79	39	22	3	25

Table 3.2.1. Host species which was investigated in this study and number of samples in each species. * Feeder mice which were sold as snake foods in the pet shop.

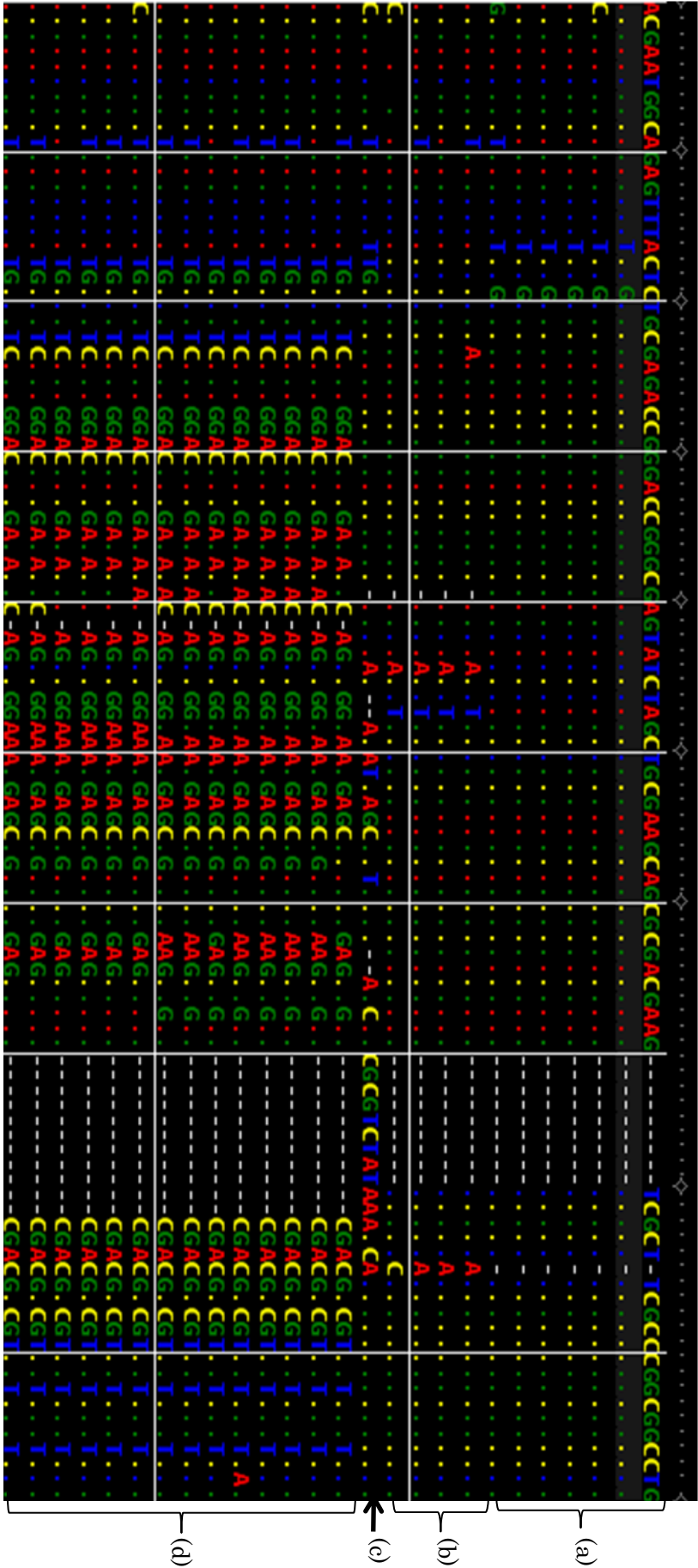


Figure 3.3.1. Sequence of pan-TTV PCR products (untranslated region) in wild rodents. Four genogroups were observed and these genotypes were tentatively called as genogroup 1a, 1b, 1c, and 2. (a) genogroup 1a, (b) genogroup 1b, (c) genogroup 1c, and (d) genogroup 2. Genogroup 1a, b, and c showed relatively similar sequences.

Pan-TTV primers applied in this study have been previously used (Thom *et al.*, 2003) and these hemi-nested primers were designed based on three highly conserved sequences in the UTR. When PCR was performed using pan-TTV primers, TTV DNA was detected in liver or spleen from 116 (92%) of 126 wood mice, 3 (7.6%) of 39 bank voles, 48 (60%) of 80 field voles, and 0 (0%) of 28 lab/house mice. The amplified products by PCR with pan-TTV primers were sequenced from 13 animals (7 wood mice, 5 field voles, 1 bank vole) testing positive and measured 77-82 bp when primer sequences were removed from both ends. To check RoTTV infection in lab/house mouse, 4 lab mice and 25 feeder mice from a pet shop were examined. However, RoTTV infection was not observed in lab/house mouse colonies.

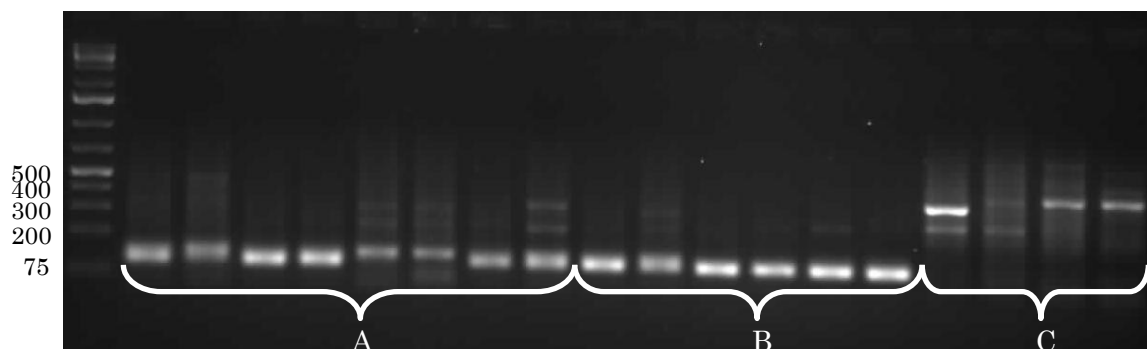


Figure 3.2.2. Screening PCR by using pan-TTV primers. (A) RoTTV positive wood mice, (B) RoTTV positive field voles, (C) RoTTV negative house/lab mice. RoTTV positive wood mice and field voles PCR results were shown as the positive controls. PCR positive bands were detected around 100 bp but house/lab mice samples were negative for pan-TTV PCR.

3.3. Sequence results of screening PCR amplicons

Pan-TTV PCR products from section 3.2. were cloned and sequenced and these sequence results were analyzed. Sequence results of pan-TTV amplicons are shown in Fig. 3.3.1. Four different genogroups were observed and these sequences were tentatively called genogroup 1a, 1b, 1c, and 2, because genogroup 1a, b, and c showed relatively similar sequences. Genetic distribution in each rodent species is shown Table 3.3.1. Genogroup 1a was observed in wood mice, bank voles, and field voles. Genogroup 2 was found in wood mice and bank voles. These findings indicated that RoTTV has extremely high sequence divergence and several genogroups were observed in same individual.

Genogroup	Rodent species
1a	Wood mice/Bank voles/ Field voles
1b	Field voles
1c	Bank voles
2	Wood mice/Bank voles

Table 3.3.1. Genetic distribution in each wild rodent species. One specific RoTTV genogroup was distributed in different host species.

3.4. Amplification of RoTTV full-length genomes

Based on common 77-82bp amplicons, distinct RoTTV sequences could be seen. To determine the diversity of viruses present in the wild rodent population, inverse primers which partially overlapped each other were used for amplification of complete RoTTV genomes in each genogroup. Before running full-length PCR, the DNA extracted from the liver, spleen, serum, and faecal supernatants of 7 (3 wood mice, 3 field voles and 1 bank vole) viraemic animals were amplified by RCA. RCA is sequence independent circular DNA amplification method by using phi 29 DNA polymerase (section 2.10.2). The full-length genomes of RoTTV were amplified by PCR with inverted-primers which are shown in Table 2.5.1. RoTTV genogroup 1a genomes were

amplified from 4 wood mice and 1 field vole and were found to be approximately 2.2kb (Lane A and B of Fig. 3.4.1). RoTTV genogroup 1b and 1c TTV genomes were amplified from 3 field voles and 1 bank vole respectively and were also observed to be approximately 2.2kb (Lane C and D of Fig. 3.4.1). RoTTV genogroup 2 genome size was slightly larger than that of RoTTV genogroup 1 suggesting these are distinct groups. RoTTV genogroup 2 genomes amplified from 3 wood mice and 1 field vole and were shown to be approximately 2.5kb (Lane E of Fig. 3.4.1). Then, PCR amplicons from each of the wood mouse, field vole and bank vole samples with the strongest PCR signal were molecularly cloned. The details of the cloned genomes are shown in Table 3.4.1. A total of 31 clones (13 genogroup 1a, 4 genogroup 1b, 1 genogroup 1c, and 13 genogroup 2) were sequenced over the complete genome.

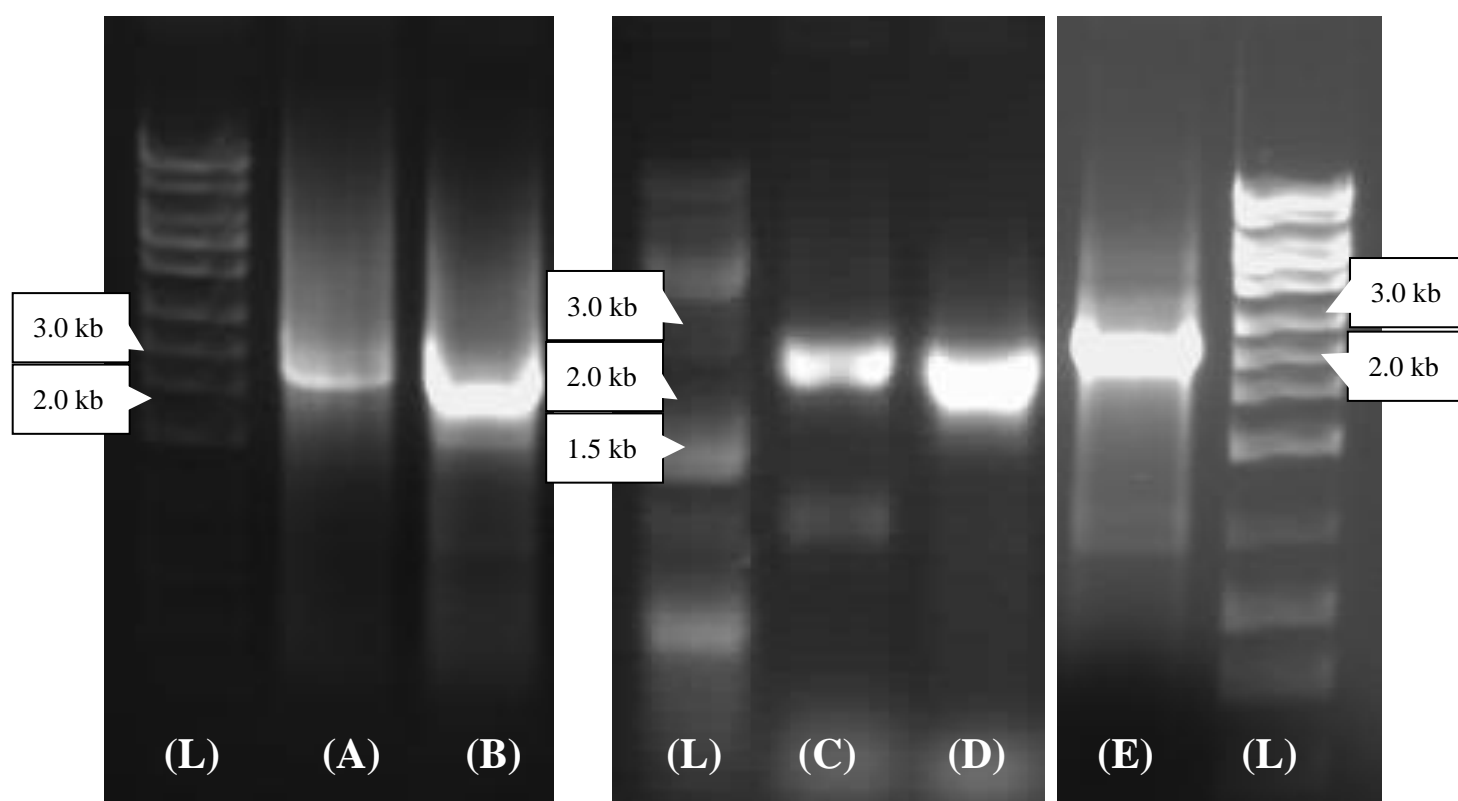


Figure 3.4.1. Full-length PCR of RoTTV. (L) DNA Ladder. (A) Genogroup 1a complete genome from spleen. (B) Genogroup 1a complete genome from spleen serum. (C) Genogroup1b from liver. (D) Genogroup1c from liver. (E) Genogroup 2 from liver. Genogroup1a, Genogroup1b, and Genogroup1c genome sizes were approximately 2.2kb. Genogroup 2 genome size was about 2.5 kb.

Species	Source	Primer set**	Number
Wood mice	WM1 spleen	1a	1 clones
	WM1 serum	1a	3 clones
	WM1 spleen	2	1 clone
	1012 spleen *	1a	1 clone
	1014 spleen *	1a	1 clone
	1012 faeces *	2	2 clones
	1012 spleen*	2	1 clone
	1014 faeces *	2	1 clone
	1014 spleen *	2	1 clone
Field voles	ML89 Liver	1a	2 clones
	ML89 Liver	2	3 clones
	ML89 Liver	2b	4 clones
	ML79 Liver	1b	2 clones
	ML80 Liver	1b	1 clone
	ML89 Liver	1b	1 clone
Bank voles	ML211 Liver	1c	1 clone

Table 3.4.1. Genomes cloned in this study.

*Complete genome was constructed from faeces and spleen in the same individual.

** Genogroups which were used for designing the primer. PCR amplicons may not be same genogroup with primer genogroups.

3.5. Complete sequence and phylogenetic analysis of RoTTV

Thirty-two complete RoTTV genomic sequences from three rodent species are shown on the CD-ROM. Each TTV genome size is shown in Table 3.5.1 (RoTTV1) and Table 3.5.2. (RoTTV2). The 17 RoTTV1 genomes ranged in size from 2164 to 2318 nt. The 13 RoTTV2 genomes were 2571 nt in size. Some field vole clones amplified using genogroup 1a primers, showed genogroup 1b sequence. RoTTV genomic sequences from spleen were identical to those from faecal supernatant in the same individual (1012 and 1014)

RoTTV ORF1 sequences were extracted based on ORF finder. The RoTTV ORF1 sequences along with 164 available ORF1s of anellovirus were aligned using MUSCLE version 3.8 in Simmonic Sequence Editor v1.6 software (section 2.8.2.) and the region of highest homology (equivalent to aa 42-241 of AS_WM1_Sp_1 ORF1) was used for the construction of a phylogenetic tree and pairwise-comparison analysis. A phylogenetic tree of representatives from RoTTVs human TTV genogroups, TTMV, TTMDV and other animal TTV is shown in Fig. 3.5.1. RoTTV showed two distinctive groups corresponding to the tentative genogroup 1 (a, b, and c) and genogroup 2 classification and will subsequently be referred to as RoTTV1 (a, b, and c) and RoTTV2. RoTTV2 sequences were similar in different species but RoTTV1 sequences differed among host species.

Pairwise-distance comparisons of aa sequence across the region described above of all available human and chimpanzee anellovirus (TTV; n=129, TTMV; n=14, TTMDV; n=21) and RoTTV ORF1s (RoTTV1; n=17, RoTTV2; n=14) are shown in Table 3.5.3. Sequence divergences of 18.7 % and 2.5 % were observed within genogroup 1 and genogroup 2 sequences respectively. However, 74.5% sequence divergence was found between these groups which are comparable the difference between these groups and other established anellovirus genera and is greater than the

divergence seen between established genera (eg TTV vs TTMV or TTV vs TTMDV). Therefore, RoTTV1 and RoTTV2 can be classified as distinct species and the divergence is even enough to potentially classify them as distinct genera pending review by the ICTV.

3.6. Genomic structure and coding capacity

RoTTV genomic maps were constructed by using DNASTAR Lasergene 9 core Suite and NCBI ORF Finder. All constructed genomic structures are shown on CD-ROM. Each ORF was analysed to find the predicted methionine start codon and a predicted protein size (more than 70 aa). All RoTTV genomic maps are shown in Fig.3.6.1. In the RoTTV genomic structures, three types of RoTTV1 genomic structures and two types of RoTTV2 genomic structure were observed. All clones had TTV ORF2 and ORF1 and RoTTV ORF2 was located in the beginning of ORF1. Genogroup 1a contained ORF2 (115aa), ORF1 (342aa), ORF3 (145aa), and ORF4 (83aa) (Fig.3.6.1. RoTTV1 structure 1). The same genomic structure was found in 11 RoTTV1 clones. Other RoTTV1 group clones also had the same genomic structure but ORF sizes were slightly different. Two clones had short ORF2 (94aa), ORF1 (346aa), short ORF3 (123aa) and long ORF4 (127aa) compared to the original clone (Fig.3.6.1. RoTTV1 structure 2). Four clones had slightly different structures from original clones and they have ORF2 (110aa), ORF1 (344aa), ORF3 (137aa), and ORF4 (86aa) (Fig.3.6.1. RoTTV1 structure 3). In contrast, RoTTV2 has relatively conserved ORF sizes with only a small difference among RoTTV2 clones. RoTTV2 consistently had ORF2 (75aa), and ORF1 (576aa). Standard RoTTV2 ORF3 had 128aa, but one group which had two clones had shorter ORF3 (83aa) (Fig.3.6.1. RoTTV2 structure 2). In addition, 2 ORFs with coding capacities of 156 and 147 aa but no methionine were also found and are called ORF4 and ORF5 respectively.

Clone No. (bp)	ORF2	ORF1	ORF3	ORF4
WM1_Sp_1 (2236)	125-472 (115)	259-1287 (342)	1271-1708 (145)	1324-1575 (83)
WM1_Se_1 (2238)	125-472 (115)	259-1287 (342)	1271-1708 (145)	1324-1575 (83)
WM1_Se_2 (2318)	125-472 (115)	259-1287 (342)	1271-1708 (145)	1324-1575 (83)
WM1_Se_3 (2318)	125-472 (115)	259-1287 (342)	1271-1708 (145)	1324-1575 (83)
1012_Fae_1 (2250)	125-472 (115)	259-1287 (342)	1271-1696 (141)	1312-1563 (83)
1012_Fae_2 (2249)	125-472 (115)	259-1290 (343)	1271-1708 (145)	1324-1575 (83)
1012_Sp_1 (2248)	125-472 (115)	259-1290 (343)	1271-1708 (145)	1324-1575 (83)
1014_Fae_1 (2235)	125-472 (115)	259-1287 (342)	1271-1708 (145)	1324-1575 (83)
1014_Fae_2 (2239)	125-472 (115)	259-1287 (342)	1271-1708 (145)	1324-1575 (83)
1014_Sp_1 (2238)	125-472 (115)	259-1287 (342)	1271-1708 (145)	1324-1575 (83)
ML89_Li_1 (2266)	128-412 (94)	262-1302 (346)	1280-1651 (123)	1342-1725 (127)
ML89_Li_2 (2266)	128-412 (94)	262-1302 (346)	1280-1651 (123)	1342-1725 (127)
ML79_Li_1 (2182)	80-412 (110)	262-1296 (344)	1280-1693 (137)	1336-1596 (86)
ML79_Li_2 (2248)	80-412 (110)	262-1296 (344)	1280-1693 (137)	1336-1596 (86)
ML80_Li_1 (2246)	80-412 (110)	262-1296 (344)	1280-1693 (137)	1336-1596 (86)
ML89_Li_4 (2164)	80-412 (110)	262-1296 (344)	1280-1693 (137)	1336-1596 (86)
ML211_Li_1(2259)	132-416 (94)	269-1309 (346)	1284-1715 (143)	1349-1600 (83)

Table 3.5.1. RoTTV1 genomic sizes and structure. Nucleotide coordinates for each ORF are listed with aa size in parentheses.

bp=base pair

Clone No. (bp)	ORF2(bp)	ORF1(bp)	ORF3(bp)	ORF4(bp)	ORF5(bp)
WM1_Se_4 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
ML89_Li_4 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
ML89_Li_5 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
ML89_Li_6 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
ML89_Li_7 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
ML89_Li_8 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
ML89_Li_9 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
ML89_Li_10 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
1012_Fae_3 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
1012_Fae_4 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
1012_Sp_2 (2571)	125-352 (75)	238-1968 (576)	1272-1658 (128)	1460-1930 (156)	1683-2126 (147)
1014_Sp_2 (2571)	124-351 (75)	237-1967 (576)	1406-1657 (83)	1459-1929 (156)	1682-2125 (147)
1014_Fae_3 (2571)	124-351 (75)	237-1967 (576)	1406-1657 (83)	1459-1929 (156)	1682-2125 (147)

Table 3.5.2. RoTTV2 genomic sizes and structure. Nucleotide coordinates for each ORF are listed with aa size in parentheses.

bp=base pair

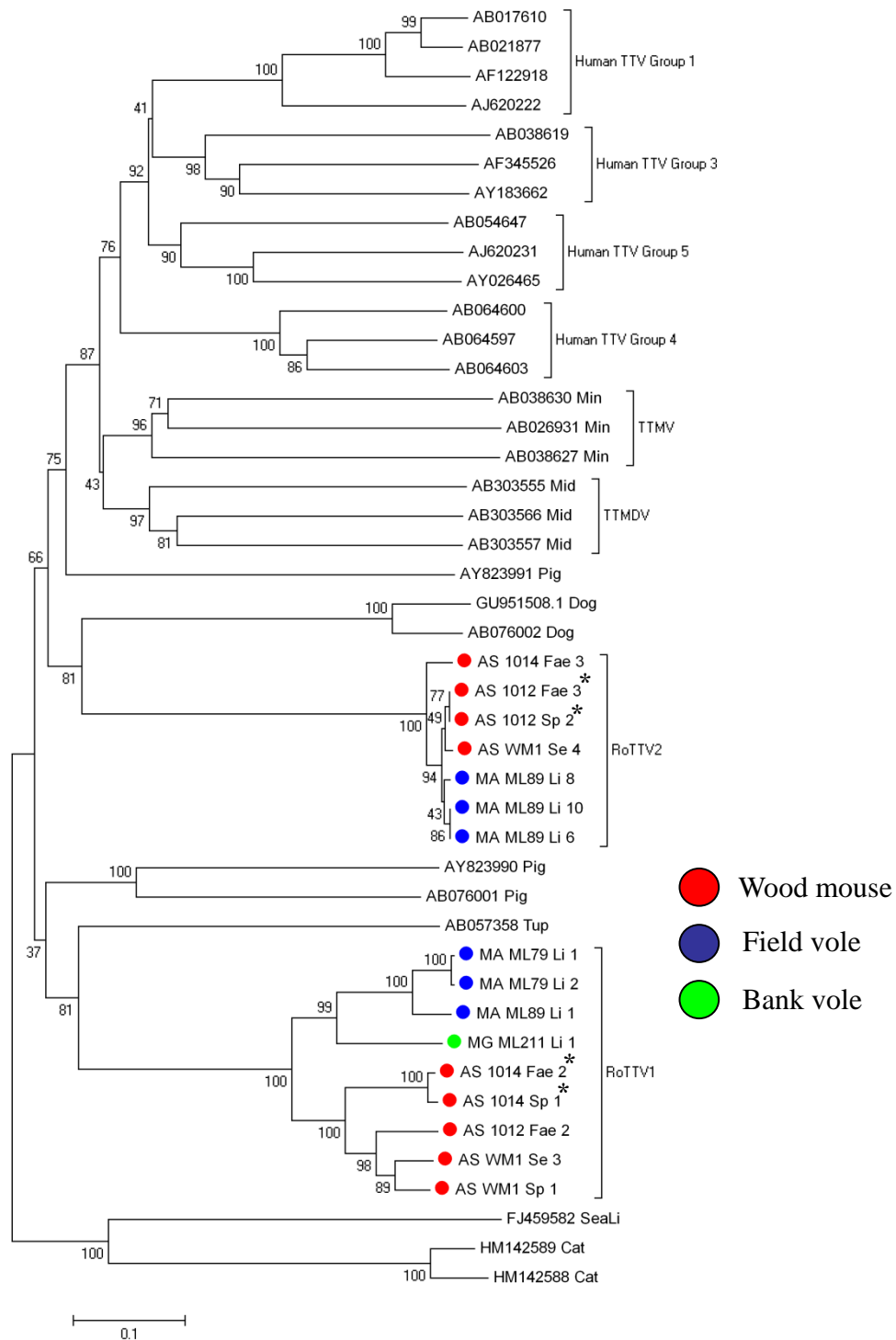


Figure 3.5.1. Representatives of RoTTV and other anellovirus phylogenetic tree based on partial ORF1aa sequence. In addition, RoTTV2 sequences in different species are similar. RoTTV1 sequences differ between host species. *RoTTV sequences which from faeces and spleen were identical.

	RoTTV1 (n=17)	RoTTV2 (n=14)	TTV (n=129)	TTMV (n=14)	TTMDV (n=21)
RoTTV1(n=17)	18.7%	74.5%	74.1%	77.3%	77.7%
RoTTV2(n=14)		2.5%	74.3%	75%	74.6%
TTV(n=129)			49.4%	68.2%	64.6%
TTMV(n=14)				54.4%	67.5%
TTMDV(n=21)					50.1%

Table 3.5.3. Group amino acid pairwise-distance comparisons within and among 17 RoTTV1, 13 RoTTV2, 129 TTV, 14 TTMV, and 21 TTMDV using partial ORF1 sequences.

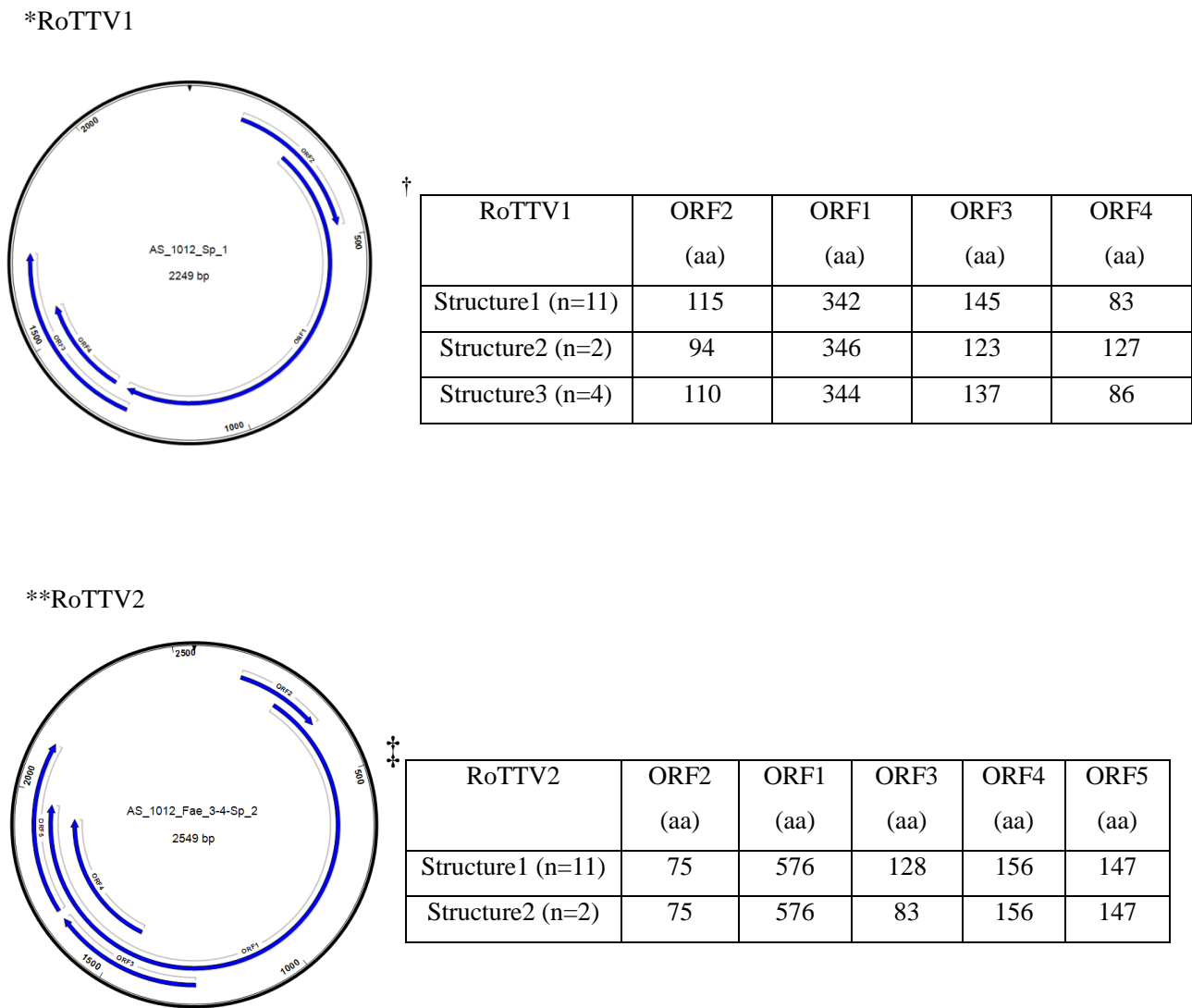


Figure 3.6.1 and Table3.6.1. RoTTV genomic maps. *Representative RoTTV1 genomic map of RoTTV. Variation is seen in the ORF sizes of different clones. †In the RoTTV1 genomes, three types of genomic structures which had different ORF were observed. ** Representative RoTTV2 genomic map of RoTTV. ‡ RoTTV2 genomic structures are shown and two RoTTV2 structures were observed.

3.7. Design of species specific primer sets for screening RoTTV genomic prevalence in wild rodents

Based on the complete sequence results, two pairs of species specific primer were designed based on ORF1 (ORF1 primer). The primers are shown in Table 2.5.1. The wild rodent populations were then screened again. The results are shown in Table 3.7.1. RoTTV1 TTV DNA was detected in liver or spleen from 101 (80%) of 126 wood mice, 3 (7.6%) of 39 bank voles, 50 (63%) of 79 field voles, and 0 (0%) of 28 lab/house mice. RoTTV2 TTV DNA was found in liver or spleen from 67 (53%) of 126 wood mice, 6 (15%) of 39 bank voles, 6 (7.6%) of 79 field voles, and 0 (0%) of 28 in lab/house mice. RoTTV2 showed low infectious prevalence compared to that of RoTTV1. Moreover, RoTTV1 TTV was widely spread in the different host species. Wood mice were frequently infected by TTV RoTTV1 and RoTTV2. In the TTV positive rodents, 37.5% (60/160) of wild rodents have RoTTV1 and RoTTV2 DNA.

	Wood mice n=126	Field voles n=79	Bank voles n=39	House mice n=28
RoTTV1 positive	101 (80%)	50(63%)	3(7.6%)	0(0%)
RoTTV2 positive	67(53%)	6(7.6%)	6(15%)	0(0%)
Coinfection rate	54 (43%)	5(6.3%)	1(2.5%)	0(0%)

Table 3.7.1. Rescreening results of RoTTV using species specific primer.

For further confirmation of the absence of RoTTV in *Mus musculus*, *Mus musculus* DNA library was constructed following section 2.10. RCA amplicons from *Mus musculus* liver DNA were digested by restriction enzyme, cloned and sequenced. Originally, RoTTV was discovered in 5 of 87 colonies from clone libraries from 2 wood mice. However, no RoTTV clones were isolated in 51 colonies from 2 *Mus musculus*. As part of the current study, an additional 102 colonies from 5 *Mus musculus* clone libraries were isolated and sequenced, but no anellovirus sequences were detected in these *Mus musculus* DNAs.

3.8. Discussion

3.8.1. Distribution of RoTTV in wild rodent population

RoTTV was discovered by using a shotgun cloning technique combined with RCA from a wood mouse sample. This RoTTV which was firstly discovered was prototype isolate and later it was confirmed as a member of RoTTV1a.

In this study, several RoTTV features were revealed and these features were quite similar to those of TTV in humans and other animals. Firstly, RoTTV showed high infectious prevalence in the wood mouse (92%) and field vole (60%) populations. RoTTV was also observed in bank voles but the prevalence was low (7.6%). No infection was seen in house/lab mice (*Mus musculus*). TTV infection has also been observed in human (Hino and Miyama, 2007; Bendinelli, 2001; Maggi and Bendinelli, 2010), non-human primates (Thomas, 1999; Thom, 2003; Okamoto, 2002), pig (Okamoto, 2002), cat (Okamoto, 2002), dog (Okamoto, 2002), tupaia (Okamoto, 2002), chicken (Thomas, 1999), cow (Thomas, 1999) and sheep (Thomas, 1999). RoTTV would be included in this TTV family. High infectious prevalence was also common in the human and swine TTV infection, and more than 80% of human and pig populations were found to be positive for TTV DNA (Hino and Miyama, 2007). In the wild rodent population, wood mice (*Apodemus sylvaticus*) RoTTV infectious prevalence was extremely high but absent in *Mus musculus*. A similar disparity in infectious status between these species has also been observed in parasites such as *Toxoplasma gondii* (Bordes *et al.*, 2012) and other viruses such as murid herpesvirus 4 (Blasdell *et al.*, 2003). Differences between the species such as immune responses and behaviour may influence the different infectious prevalence between wood mouse and other rodent species. Furthermore, we believed that every animal might harbour a TTV because TTV infection widespread in many animals. However, house/lab mice (*Mus musculus*) were found to be RoTTV negative in wild, lab, and pet shops. Compared to the other rodent species, the physiology and immunology of house/lab mice are well studied. Conversely, the physiology, especially immunology of wood mice and vole species has not been studied and only a few papers have been published. Most of the wood mice immunology papers mention that wood mice

have a strong immune system compared to *Mus musculus*. For example, wood mice showed a stronger interferon response and natural killer (NK) cell activity in Central European Encephalitis (CEE) virus infection (Kopecky *et al.*, 1991). Furthermore, neutralising antibodies of wood mice appear faster and reached higher titer in the early phase of CEE virus and murine gamma herpes virus infection (Kopecky *et al.*, 1991; Francois *et al.*, 2010). Also, parasite infection is thought of as an important immunomodulatory factor (especially innate immunity) in the wood mice (Jackson *et al.*, 2009), and these immunomodulatory systems or strong immune reaction may make a difference in their infectious status. However, this information is limited and there may be other immune system functions which are present in house/lab mice but not wood mice and vole species.

3.8.2. Co-evolution of TTV and host species

There is another possible reason why TTV infection is not observed in *Mus musculus*. For TTV evolutionary history, it has been hypothesized that co-evolution is the one of the most important factors to make the wide genetic diversity (section 1.2.). Although there are contradictory ideas, some researchers have suggested that TTV replicates slowly and takes millions of years to mutate (Khudyakov *et al.*, 2000). We predicted that RoTTV has not been adapted to *Mus musculus* and it may simply need more time to mutate for the infection. In fact, the *Mus musculus* species is not native mouse in Europe. As early as 12,000 years ago, the *Mus musculus* was observed in Middle East (Auffray *et al.*, 1988). After that, these mice spread to Asia. During the Edo era (100-400years ago), Japanese hobbyist attracted by the coat colour variants of *Mus musculus*, bred them to produce new colours and sizes of mice and set up a "fancy mouse" trade. From the mouse trade, these mice finally spread to Europe and England (Moriwaki, 1994). According to this, *Mus musculus* appeared U.K environment only in the last several hundred. Therefore, TTVs may have to adopt in order to infect the house mice because TTV is species specific virus. Thus RoTTV must evolve by mutation and it is considered that RoTTV has not mutated enough to infect to the lab/house mice. On the other hand, wood mice are sometimes used for phylogeographical study because wood mice have relatively a large population in the wild rodent

and are present in European region for the past 3 million years (Michaux *et al.*, 1998). This fact may also explain why wood mice become the natural host for the many pathogens in U.K.

3.8.3. Genetic diversity of RoTTV

RoTTV had at least 4 genogroups based on the sequence of screening PCR products and these RoTTV types are spread in different host species. Furthermore, screening PCR using species specific primers showed 37.5% (60/160) of RoTTV infected wild rodents had both RoTTV1 and RoTTV2. Multiple infections of different types of RoTTV in same individual were common. Multiple infections of several TTV genotypes have also been observed in human TTV infection (Ninomiya, 2008). TTV is thought to be acquired in humans during infancy and dual and triple TTV infection were observed in 86.9% 1 year old children.(Ninomiya *et al.*, 2008) In the swine TTV research, vertical transmission of TTV has been indicated (Pozzuto *et al.*, 2009) The early acquisition of multiple TTV infections could occur in wild rodents population.

We found that RoTTV has also a circular DNA genome and it was supported by successful amplification after RCA and inverted PCR. After sequencing of RoTTV complete genomes, we found that RoTTV showed heterogeneity of the entire viral genome sequences and sizes. For the phylogenetic tree construction, representatives of TTV, TTMV, TTMDV, animal TTVs, and RoTTVs were used and high sequence divergence was shown between RoTTV1 and RoTTV2, comparable to the difference between RoTTV and other anellovirus genera in pair-wise comparison analysis. Therefore, RoTTV strains could be divided into two species (RoTTV1 and RoTTV2), potentially even into different genera and each species had several genotypes. This genetic diversity is also common in human and animal TTV infection. Based on the wide range of human TTV sequence divergence, human TTV has at least 39 different genotypes, exhibiting >30% nucleotide difference from one another, and 5 different groups, showing >50% divergence (Okamoto *et al.*, 2004). Therefore, RoTTV genogroups have a similarly high genetic diversity among the each genogroup compared to human TTV. In addition, RoTTV1 and RoTTV2 were found in the different rodent species; wood mice, field voles and bank voles. Also, RoTTV1

genomic sequence differs among the different host species than those of RoTTV2. This is unusual because other TTVs are considered to be species specific and would not show cross-species infection (Isaeva and Viazov, 2002; Brassard *et al.*, 2008; Okamoto *et al.*, 2002; Thom *et al.*, 2003). However, the similar infective style which makes a cross-species infection only among the rodent species is also observed in murid gammaherpes virus infection (Francois *et al.*, 2010). Herpesviruses are also known to be highly host restricted but murid gammaherpesvirus infection is observed in a wide variety of hosts including mouse, shrew and bank vole (McGeoch *et al.*, 2006). According to several studies, it is concluded that this intraspecies transfer could be a key factor of gammaherpesvirus evolution (Wibbelt *et al.*, 2007; Prepens *et al.*, 2007; Ehlers *et al.*, 2007; Ehlers *et al.*, 2008). It is assumed that the reason why gammaherpesvirus transmits among the small rodents rather than large mammals is simply that small rodent species share the same habitat. Moreover, because of the horizontal transmission between small rodents, murid gammaherpesvirus sequences change rapidly compared to other members of *gammaherpesvirinae* (McGeoch *et al.*, 2001; McGeoch *et al.*, 2005). For intraspecies infection, murid gammaherpesvirus could be forced to adjust to new host receptors and immune system and one of the essential factors could be fast mutation (see below). This may also explain the finding that RoTTV showed cross-species infection in the different small rodent species and extraordinary sequence variations.

We have several suggestions about the diversity of RoTTV. Firstly, TTV is a ss DNA virus. ssDNA viruses have a higher mutation rate compared to dsDNA viruses, even as high as some RNA viruses (Duffy, 2008). It is thought that ssDNA have high substitution rates (Duffy, 2008). Secondly, smaller viruses like TTV tend to replicate faster which increases the chance for accumulated virus mutations (Duffy, 2008). Thirdly, TTV is thought to use direct infection and does not use a vector (Lin *et al.*, 2000). Therefore, TTV has to infect new host by itself and they may be required to mutate to apply to a new environment. Fourth, viruses which use the horizontal infection have to infect new individuals, thus these viruses may mutate to adjust the different individuals and their replication speed is faster than the viruses which infect vertically

(Lin *et al.*, 2000). Moreover, TTV high mutation frequency was observed in human TTV experiments. In a cell culture experiment, TTV mutation and genetic rearrangement were frequently observed in TTV infected cells (Lippik, 2007). The high mutation rate and TTV genomic rearrangement may be related to the RoTTV genetic divergence.

RoTTV complete genome sequences from spleen were identical with those from faecal supernatant. This indicated that RoTTV was emitted in the faeces and faecal supernatant is a good tool for the diagnosis of TTV. Furthermore, faeces would be an important route for TTV transmission. TTV was originally called transfusion transmitted virus because TTV was thought to be transmitted via blood transfusion (Takahashi *et al.*, 2000). However, transmission via blood cannot explain the high infectious prevalence of TTV. As a result, the faecal-oral route was believed to be the main route of TTV infection. These results in this study also support this hypothesis. Similar results were also observed in swine and cattle TTV research (Brassard, 2008).

3.8.4. Genomic structure of RoTTV

RoTTV genomic structure was similar to that of TTV in humans and other animals. Genomic length of RoTTV was much smaller than those of other human and animal TTV. In human infection, 3 sizes of anellovirus were observed; TTV, TTMDV, and TTMV. TTV which was originally discovered is 3.8kb (Hino and Miyata 2007). TTMDV and TTMV have a smaller size compared to TTV, 3.2kb and 2.8kb respectively (Hino and Miyata 2007). On the other hand, RoTTV1 was 2.2kb and this is almost same genome size as tupaia TTV (Okamoto *et al.*, 2002). RoTTV2 was 2.5kb. It has been shown that all animal isolates were smaller than human isolates, but to compared the previous TTV study, RoTTV genomic size was different from those of human and other animal TTV genomes. RoTTV genome sizes were between human TTMV (2.8kb) and feline TTV (2.0kb) and these sizes were uncommon except for tupaia (Okamoto 2002). However, an anellovirus, which has same size as RoTTV, was observed in mosquito. These viruses were called as mosquito anellovirus and their genome sizes were 2.3kb and 2.5kb which is almost the same size as RoTTV1 and RoTTV2 respectively. We hypothesise that the

mosquito is not the true host of mosquito anellovirus and rodents are the source of anellovirus in mosquitoes. This idea is also supported by evidence that TTV isolated from rats and rat TTV showed to 81% mosquito anellovirus genomes (see Appendix 1.)

The complete preservation of coding capacity for ORF1, ORF2, ORF3 and ORF4 was illustrated in Fig.3.6.1. Although RoTTV genome sequence and size were variable, the genomic organisation resembles those of TTV, TTMDV, and TTMV which were isolated from humans, nonhuman primates and other domestic animals. RoTTV ORF2 was located just before ORF1 and RoTTV RoTTV1 ORF2 was 50% identical to swine TTV ORF2. RoTTV2 ORF2 was 84% identical with mosquito anellovirus ORF2. Interestingly, mosquito anellovirus was 80% identical to RoTTV1 ORF1 and 89% identical to RoTTV2 ORF1. These analogies of sequences between mosquito anellovirus and RoTTV indicate that RoTTV is closely related to mosquito anellovirus. Mosquito anellovirus was discovered in mosquitos in San Diego County (Nq *et al.*, 2011). Mosquitos are an important vector and reservoir of various virus populations. In the present study, it is indicated that wild rodent species share similar TTV with mosquito. After ORF1, there were one or two ORFs in RoTTV1 and RoTTV2. According to the predicted genomic organization of other TTV genome, ORF3 and ORF4 should be located after ORF1 (Okamoto, 2002). Thus, we believed these two ORFs would be ORF3 and ORF4 respectively. In the RoTTV2 genome structure, structure 1 had an antisense ORF (the data was not shown). TTV is described as single stranded negative sense DNA virus (Okamoto *et al.*, 2009). Therefore, this antisense ORF which was often observed in RoTTV2 should not exist. This antisense ORF has no TATA box and poly A tail. This fact also supports that RoTTV2 TTV has antisense ORF but likely is not translated or transcribed. Finally, this antisense has to be checked by using northern blot. Thus, this antisense ORF has to be analyzed carefully.

3.8.5. Classification of RoTTV

In this study, 31 RoTTV1 and RoTTV2 isolates were totally separated from CAV which is most closely related to TTVs among known human and animal viruses. Basic Local Alignment Search

Tool (BLAST) analysis revealed that RoTTV1 and RoTTV2 isolates were closer to mosquito anellovirus (Nq *et al.*, 2011). The genomic length of TTV tends to be smaller in the lower order animals. In this study, we can support this idea because RoTTV was much smaller than any other human and animal TTV except for feline TTV (Okamoto *et al.*, 2002). At present, there are no precise criteria to distinguish between TTV and TTMV. Therefore, RoTTV might be called as rodent TTMV based on the genome size, but in the interim, we would propose these viruses as RoTTV. This is consistent with other anelloviruses being called TTV in other animal species.

TTV has many unknown characteristics such as pathogenesis, virus localization, and host immune response. Current TTV research has been limited by the lack of good animal model and cell culture system. RoTTV has the potential to be an ideal animal model for TTV research.

In conclusion, RoTTV was discovered in wild rodent population and, in spite of sequence divergence, the genomic organization was similar to TTV in human and other animals. Further studies of RoTTV are required for an understanding of RoTTV pathogenesis and vorology.

Appendix 1. Rat TTV infection in lab rat

A.1. Background to this study

To look for other potential animal models of TTV infection, RoTTV distribution in lab rats was studied. By finding RoTTV in the lab rats, we could also identify the virus contamination in the lab animal. If TTV is widespread in the lab rat colonies, this virus may disturb the rat immune system and affect the outcome of other experiments involving these animals.

A.2. Samples

Colony 1: two rats (*Rattus norvegicus*) were euthanized by cervical dislocation and sera were taken. Colony 2: ten rats were euthanized by CO₂. Livers and spleens from these rats were taken.

A.3. Screening PCR by using pan-TTV primer

Screening PCR was carried out using the pan-TTV primer set which was used for screening wild RoTTV. When PCR was performed using the pan-TTV primers, TTV DNA was detected in serum from 2 rats (100%) and liver or spleen from 10 rats (100%). The amplified products by PCR with pan-TTV primers, which measured 54bp (primer sequence at both ends removed) were sequenced for the 12 rats which tested positive and analysed. According to the sequence results, 12 rats TTVs showed 96-98% sequence identified to each other.

A.4. Full-length PCR to amplify rat TTV

Based on common 54bp sequences, inverse primers Ratinv1 (sense 5' CGG STA TGC CTG GTC CTC C 3') and Rat inv2 (antisense 5' CGG GAA GCG AAG CGC CGA C 3') for amplification of rat TTV in each were constructed to amplify the full-length genomes. Before running full-length PCR, DNA extracted from the liver of 4 viraemic rats was amplified by the RCA method. Rat TTV genomes were found to be approximately 2.5kb in size in 4 rats Fig. A4.1. These amplified rat TTV genomes were cloned and sequenced.

A.5. Genomic structure and coding capacity

RoTTV genomic maps were constructed by using DNASTAR Lasergene 9 core Suite and NCBI ORF Finder. All RoTTV genomic maps are shown in Fig.A5.1. Rat TTV genomic structure was similar among the each rat TTV clones and it was also similar to those of RoTTV from the wood mice and voles. All clones had TTV ORF2 and ORF1 and rat TTV ORF2 was located upstream of ORF1 with a partial overlap. Rat TTV contained ORF1 (79aa), ORF2 (396aa), ORF3 (218aa), and ORF4 (132aa).

A.6. Discussion

TTV has been discovered in almost every animal population. However, TTV in rodents had not been described until the discovery of RoTTV in from wood mice. This suggested that rat TTV might exist in wild or lab rats. Interestingly, all 12 rats were positive using the screening PCR. In this study, there were 2 groups and they come from two different labs. Although some rats were separated in different cages, the 10 rat group all came from the same animal house. The high infectious prevalence might be caused by virus propagation in the colony. However, the other two rats came from a totally different colony but they shared a quite similar virus. This indicates that rat TTV is widely distributed in lab rat population. Rat TTV genomic structure was similar to TTV from the other animals including RoTTV from wood mice and voles. Surprisingly, the rat virus complete genome sequence was 81% identical to mosquito anellovirus complete genomes. This sequence result also supports the hypothesis that rodent species provides TTV to mosquito and the mosquitoes are not natural host of TTV (section 3.8.4.).

The rat is also an important lab animal and many research reagents are available for rat experiments. Rat TTV is relatively easy to be detected in the rat. However, this means control rats may be difficult to find in the rats colonies. Epidemiological study would be required to understand the rat TTV distribution.

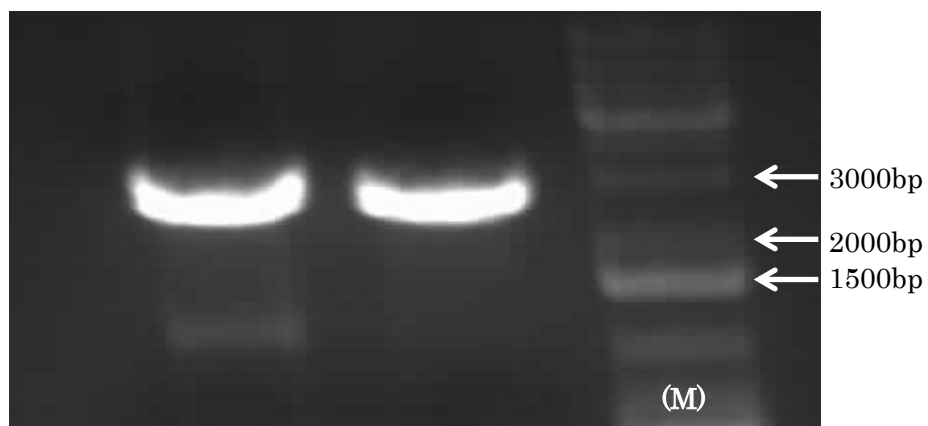


Figure A4.1.1. Full-length PCR of rat TTV. (M) DNA ladder. Rat TTV complete genome was observed to be approximately 2.5kb in size.

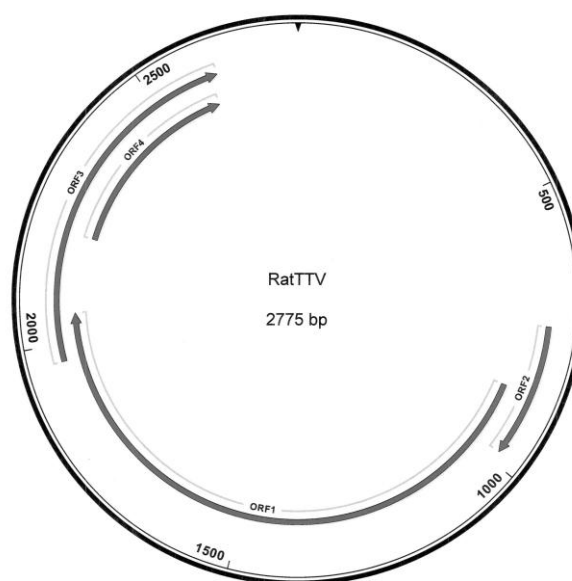


Figure A.5.1. Rat TTV genomic structure. Rat TTV genomic structure was similar to RoTTV1. All rat TTV genome structure was similar. ORF2 was located before ORF1.

CHAPTER IV. TRANSCRIPTS IDENTIFICATION AND PROTEIN EXPRESSION OF RODENT TORQUE TENO VIRUS

The cell types supporting TTV replication are still unknown. This information is important not only for understanding of TTV pathogenesis but also for establishing a successful TTV cell culture system. TTV infected wood mice were examined to study virus RNA distribution and transcript profile which was then applied to the protein expression in BHK-21 cells.

4.1. Background to the study

4.2. TTV RNA detection in systemic organ of RoTTV infected wild rodents

4.3. RACE (Rapid amplification of cDNA ends) and transcripts of RoTTV

4.4. Characterisation of the spliced TTV mRNAs and genomic organization

4.5. Predicted proteins and subcellular localisation of RoTTV fusion proteins

4.6. Apoptosis inducing capacity of RoTTV proteins

4.7. Confirmation of viral protein by Western blot

4.8. Discussion

4.1. Background to the study

TTV DNA is present in almost every organ and tissue and it may indicate TTV has a wide cell tropism (Reviewed by Hino and Miyata. 2007). There are many candidates for the replication site of TTV because double stranded circular DNA, which is potentially replicating virus, has been observed in human lung (Bando *et al.*, 2001), liver (Okamoto *et al.*, 2000a), bone marrow (Okamoto *et al.*, 2000b), stimulated peripheral blood mononuclear cells (PBMC) (Mariscal *et al.*, 2002), spleen, lymph node, thyroid gland, pancreas and kidney (Okamoto *et al.*, 2001). However, a TTV experiment using Rhesus monkeys showed different results. Although TTV DNA was found in various organs (Luo *et al.*, 2000) TTV replicative form was detected only in liver, bone marrow and small intestine (Xiao *et al.*, 2002). This may indicate that genetically different virus types show different tissue tropism in different host species. Alternatively, TTV could replicate in cells of the immune system and is detected throughout the tissues because of the wide distribution of these cells. Therefore, we have to carefully evaluate whether TTV really replicates in each of the tissues (Takahashi *et al.*, 2002).

As there is no efficient cell culture system to support TTV replication, the TTV transcriptional profile has been studied by using the cells transfected with genotype 1 or genotype 6 containing plasmids. Experiments have been carried out using cloned human TTV genotype 1 isolate and the monkey kidney cell line Cos-1. Three clone derived transcripts; 2.9-3.0kb, 1.2kb and 1.0kb were observed from transfected Cos-1 cells (Kamahara *et al.*, 2000; Okamoto *et al.*, 2000), and a more recent study showed one more transcript (0.6 kb) in genotype 1 transfected Huh 7 cells (Mueller *et al.*, 2008). A similar result was also observed in bone marrow cells from TTV infected patients. These mRNAs were generated as the result of splicing and in the shorter mRNA of 1.2kb, 1.0kb, and 0.6kb (Muller *et al.*, 2008) the 5' ends of ORF1 and ORF2 connect to distant ORFs to make five new ORFs (section 1.4.2.). The splice site was conserved among the different TTV genotypes (Kamahara *et al.*, 2000; Okamoto *et al.*, 2000). This indicated that TTV has several splicing sites to create the various mRNAs for various putative proteins.

Based on TTV transcripts profile, protein expression has been performed in a few studies. TTV genotype 6 showed three mRNAs and at least six predicted proteins (Qiu *et al.*, 2005). The transcriptional profile of P/1C1 (genogroup 1) was studied and four transcripts and seven predicted proteins were found (Mueller *et al.*, 2008). The proteins from these transcripts were expressed in human 293 cells and HEL32 cells and the predicted proteins were all observed (Qiu *et al.*, 2005; Mueller *et al.*, 2008) (section 1.4.3.).

In chapter III, the RoTTVs found were classified as RoTTV1 and RoTTV2, and RoTTV1 had four predicted ORFs and RoTTV2 had five predicted ORFs. However, it was not known whether the ORFs were actually transcribed. In this chapter, for understanding of the RoTTV transcripts and protein, infected wild mice were examined to study RoTTV RNA expression pattern and RoTTV transcripts. In addition the proteins were expressed based on the transcripts profiles.

4.2. TTV RNA detection in systemic organs of RoTTV infected wild rodents

There are two aims to examine RoTTV RNA from the systemic organ. One aim is finding TTV replication site could assist in setting up a cell culture system (chapter V). The other is establishing the transcriptional profile based on the extracted RNA. The wild rodent samples described in chapter III were relatively old and had been stored at -20°C. It was highly unlikely that intact RNA could be recovered from these samples. Therefore, fresh rodent tissues were required for the RNA study. Four RoTTV1 positive wood mice were caught in United Kingdom and their bone marrow, lung, liver, spleen, and kidney were taken. Total RNA was extracted from these tissues and RT-PCR was performed by using RoTTV1 specific primers (Table 2.5.1.). The PCR results are shown in Table 4.2.1. RoTTV1 RNA was not observed in every tissue, but RNA was consistently detected in bone marrow and lung tissue. Interestingly, liver was not a common site for RoTTV1 RNA in the infected wild wood mice. The RT-PCR result of mouse C is shown in Fig. 4.2.1. Mouse C showed RoTTV1 only in bone marrow and lung RNA. Bone marrow cDNA showed a stronger RoTTV1 amplicon signal than that of lung cDNA. This result was also supported by real-time RT-PCR, and RoTTV1 cDNA from bone marrow showed 1200 copies/ μ l,

but cDNA from lung was 1-4 copies/ μ l. This suggested that rodent RoTTV1 was actively replicated in the bone marrow. β -actin detection in these cDNAs confirmed the RNA integrity (Fig. 4.2.2.). This mouse C was used for the next study (section 4.3.)

Table 4.2.1. RNA distribution of RoTTV1

Case No.	RoTTV1 RNA				
	Bone marrow	Lung	Liver	Spleen	kidney
Mouse A	NS [‡]	+	—	—	—
Mouse B	NS	+	+	+	+
Mouse C	++	+	—	—	—
Mouse D	++	+	—	+	+

Results of RT-PCR for RoTTV1 ; ++ strong positive, + positive, - negative. NS=no sample

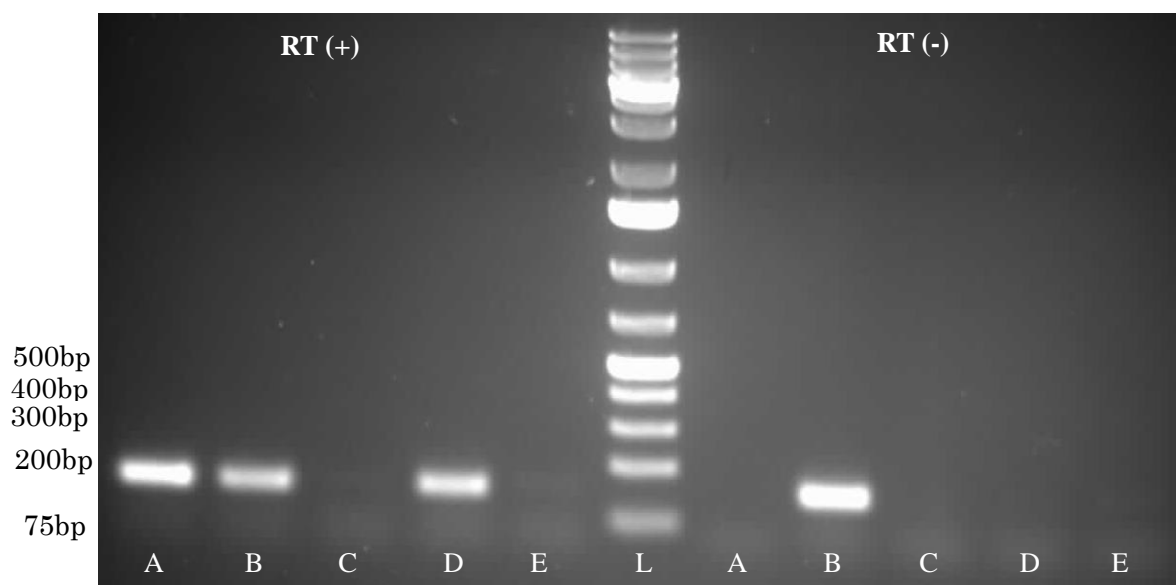


Figure 4.2.1. RT-PCR of mouse C by using RoTTV1 specific primer.

(L) DNA Ladder. (A) Bone marrow (B) spleen (C) liver (D) lung (E) kidney. RoTTV1 transcript was observed in bone marrow and lung cDNA. Bone marrow showed stronger TTV transcript expression than that of lung, and lung also showed the positive band. Spleen had positive band but it was also observed in RT (-) RT-PCR. It was later confirmed that spleen cDNA was negative by doing vigorous DNase digestion.

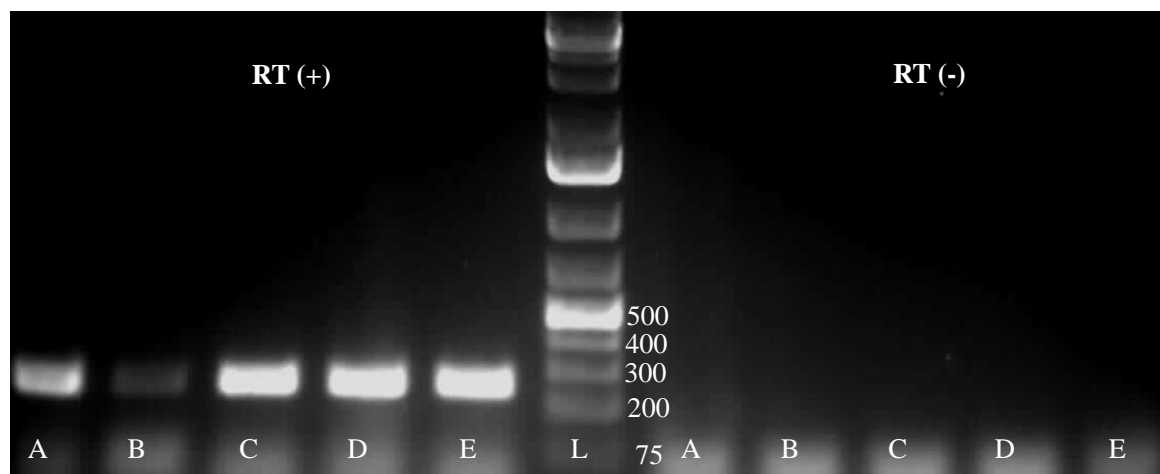


Figure. 4.2.2. RT-PCR by using β -actin primer.

(L) DNA Ladder. (A) Bone marrow (B) spleen (C) liver (D) lung (E) kidney. β -actin was observed in cDNA from these tissues and RoTTV1 cDNA was detected by using RT-PCR in Fig. 4.2.1.

4.3. RACE (Rapid amplification of cDNA ends) and transcripts of RoTTV

Full length nucleotide sequence of RoTTV1 and RoTTV2

Using DNA extracted from the RoTTV1 infected wood mouse from which the transcriptional profile was to be mapped, a complete genome was amplified and cloned for sequencing. The RoTTV1 clone was named AS_HL1_Li_1 and this clone had the same four ORFs as other RoTTV1 clones (chapter III) and a genome length of 2201 nt. The RoTTV2 complete genome, AS_WM1_Se_4 clone was obtained from the screening study from chapter III and the genomic structure was a typical RoTTV2 structure with five ORFs. The genomic maps from RoTTV1 and RoTTV2 clones are shown in Fig. 4.3.1.

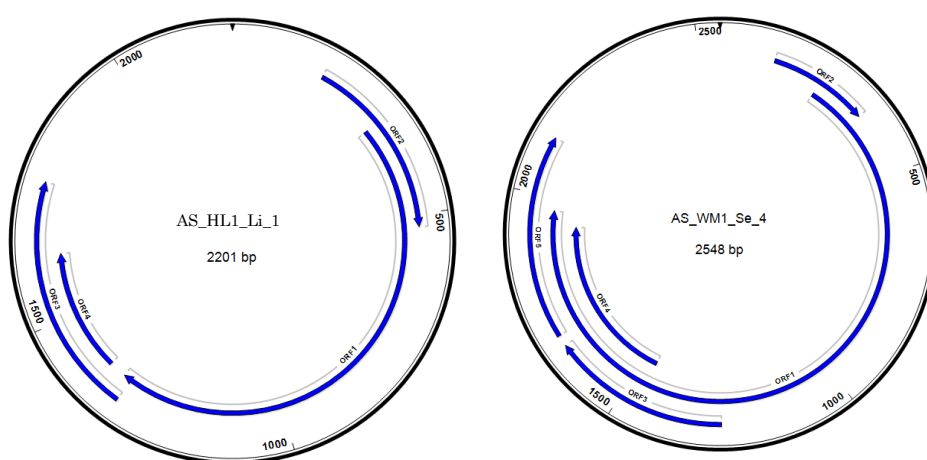


Figure 4.3.1. Genomic structure of RoTTV1 and RoTTV2 clones used in this study.

RACE (Rapid amplification of cDNA ends)

The amount of lung RNA was sufficient for RACE analysis of RoTTV1 (Rapid amplification of cDNA ends). RoTTV2 RNA was extracted from AS_WM1_Se_4 clone transfected BHK-21 cells (chapter V) for RACE analysis. By using RNA from these samples, RoTTV transcripts were identified (section 2.12.). RoTTV transcripts were amplified by using the gene specific primers (TTV primers) and GeneRACER primers (adaptor primer) shown in Table 2.12.1. Single products were amplified from 3' RACE and 5' RACE PCR respectively. For 3' RACE, the gene specific primer was located at nt 261 (see RACE primer shown in Fig. 4.3.2.A.) and the amplicon was approximately 320 bp in size. For 5' RACE, genespecific primer was located in nt 240 (see

RACE primer shown in Fig. 4.3.2.A.) and the amplicon was approximately 330 bp in size. The PCR amplicons from the RACE cDNA were cloned using the pGEM®-T Easy vector kit and sequenced. RoTTV1 3' end and 5' end sequences are shown in Fig. 4.3.2.B. The 5' terminus of RoTTV1 is predicted to start at nt 2174 with the sequence 5' AGTTTACT- downstream of TATA box (ATATAA). Also, the extreme 3'-end containing the poly A sequence -AATAAA 3' starting at nt 1699 was detected. Also, a single splicing event was observed between nt 398 and 1289. Based on RACE PCR results, two sets of primers were designed including first methionine of ORF2 and stop codon of ORF2-3 (Table 2.12.2) to confirm the transcript (see confirm primer shown in Fig.4.3.2.A). Amplicons of cDNA approximately 580 bp and approximately 1450 bp Fig. 4.3.3.A were amplified by PCR from lung cDNA representative of spliced (0.8kb) and unspliced (1.7kb) transcripts. However, only the 0.8 kb transcripts could be amplified from bone marrow cDNA (data not shown). Finally, the RoTTV1 transcript map is shown in Fig. 4.3.2.A. and shows the two distinct species of TTV mRNA, one unspliced and one spliced mRNA.

RoTTV2 transcript was also amplified by RACE PCR. Single products were amplified from 3'RACE and 5' RACE PCR respectively. For 3' RACE, the gene specific primer was located in nt 243 (see RACE primer shown in Fig. 4.3.4.A.) and the amplicon was approximately 650 bp in size. For 5' RACE, genespecific primer was located in nt 221 (see RACE primer shown in Fig. 4.3.4.A.) and the amplicon was approximately 300 bp in size. The PCR amplicons from the RACE cDNA were cloned using the pGEM®-T Easy vector kit and sequenced. RoTTV2 3' end and 5' end sequences are shown in Fig. 4.3.4.B. The 5' terminus of RoTTV2 mRNAs starts at nt 2534 with the sequence 5' CATGGACT- downstream of TATA box (TATATAA). Also, the extreme 3'-end containing the poly A sequence -AATAAAAA 3' starting at nt 2127 was detected. Also, single splicing was observed between nt 348 and 1703. To confirm the results, the PCR was performed by using 3' gene specific primer and the primers designed based immediately downstream of poly A tail (Table 2.12.2) (see confirm primer show in Fig.4.3.4.A); the amplified products of cDNA c (100-200 bp) (Fig. 4.3.3.B, product-c) and cDNA d (700-900 bp) (Fig. 4.3.3. B, product-d) were observed on 1.5% agarose gel. The sequences from PCR products were

identical to those of cDNA sequences from RACE and furthermore, several other spliced transcripts were found from the PCR. The RoTTV2 transcripts map is shown in Fig. 4.3.4.A. and shows the five distinct species of TTV mRNA with one unspliced mRNA and four different spliced transcripts. In the four spliced mRNAs, the splicing events were found to occur between nt 348 and nt 1703 (0.77 kb), nt 348 and nt 2049 (0.4 kb), nt 336 and nt 1984 (0.47kb), nt 356 and nt 2008 (0.47kb). The length of these five mRNAs was deduced to be approximately 2.1 kb, 0.77 kb, 0.4 kb, 0.47 kb, and 0.47 kb respectively by sequence analysis of the corresponding cDNA from RACE.

Without prior reverse transcription, no PCR reaction was observed using any of the primers shown in Table 2.12.2. This indicated that the above-mentioned PCR products truly come from the RNA molecules suggesting that, as predicted in chapter III, the ORFs in 3'-5' genomic orientation are not transcribed. TTV genome has been demonstrated as negative sense single stranded DNA virus.

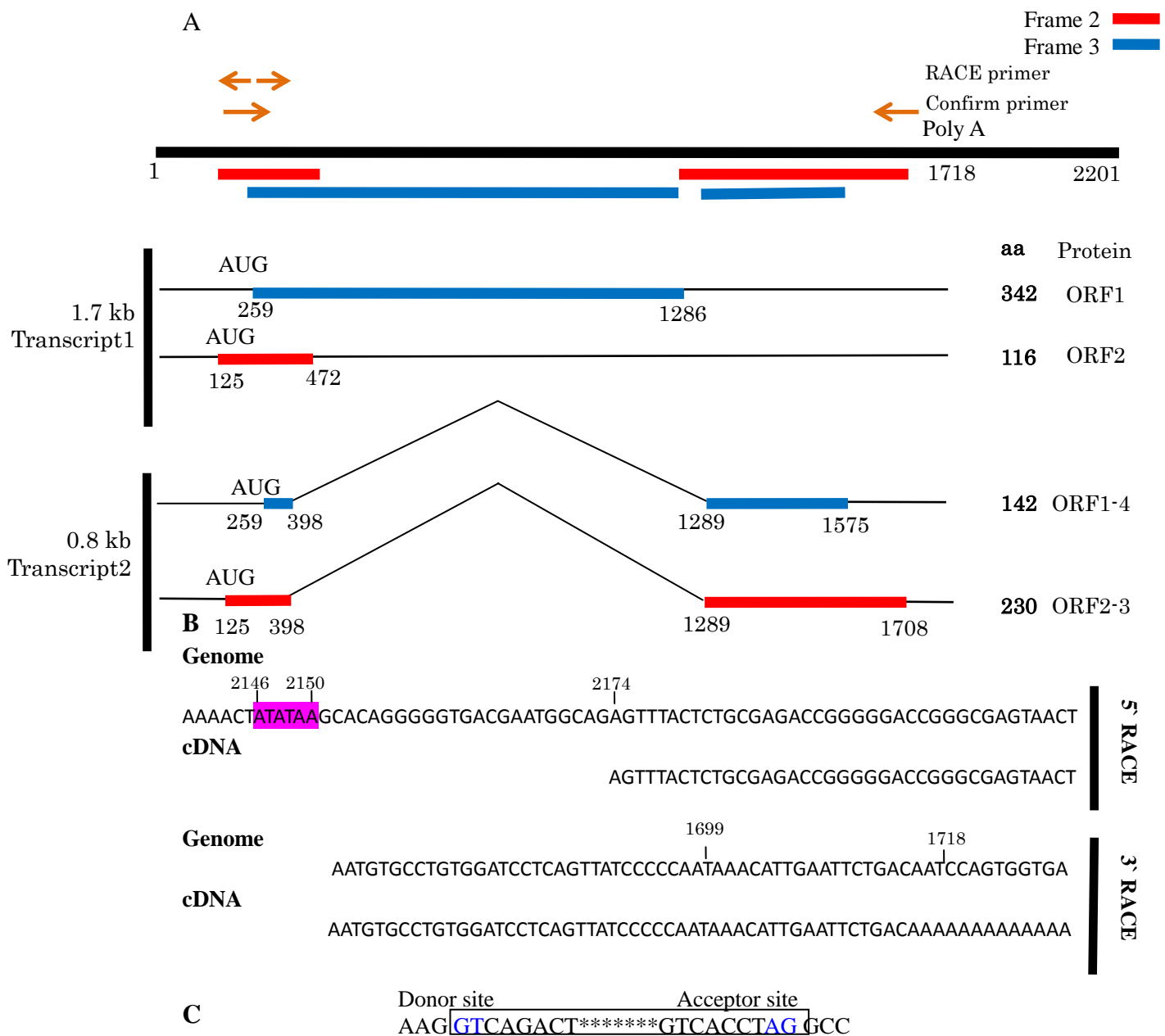


Figure 4.3.2. cDNA analysis of RoTTV1 (clone; AS_HL1_Li_1). (A) Summary of the RoTTV1 transcriptional map. The genetic map of RoTTV1 is shown on the top. The nucleotide numbers initiation site and polyadenylation cleavage site are indicated. In addition, the primer sites of Race and conformation PCR were indicated as orange arrow. Four of the predicted ORFs are diagrammed below. The ORF1 and ORF2 proteins are encoded from the unspliced 1.7 kb mRNA by using AUG (nt 259) and AUG (nt 125), respectively. ORF1-4 which is initiated at AUG (nt 259) and ORF2-3 which is initiated at AUG (nt 125) are encoded from the single spliced 0.8 kb mRNA. (B) Mapping of 5' and 3' end of RoTTV1 (clone AS_HL1_Li_1) transcripts. Mapping of 5' end of the RoTTV1 (clone AS_HL1_Li_1) transcripts and the genomic sequence with TATA box are given above. Mapping at the 3' end of the RoTTV1 (clone AS_HL1_Li_1) transcripts with poly A (nt 1718) is shown. (C) Sequence of the donor and acceptor site in RoTTV1 (clone AS_HL1_Li_1). The consensus sequence of splice donor (SD) and splice acceptor (SA) sites in splicing site is shown.

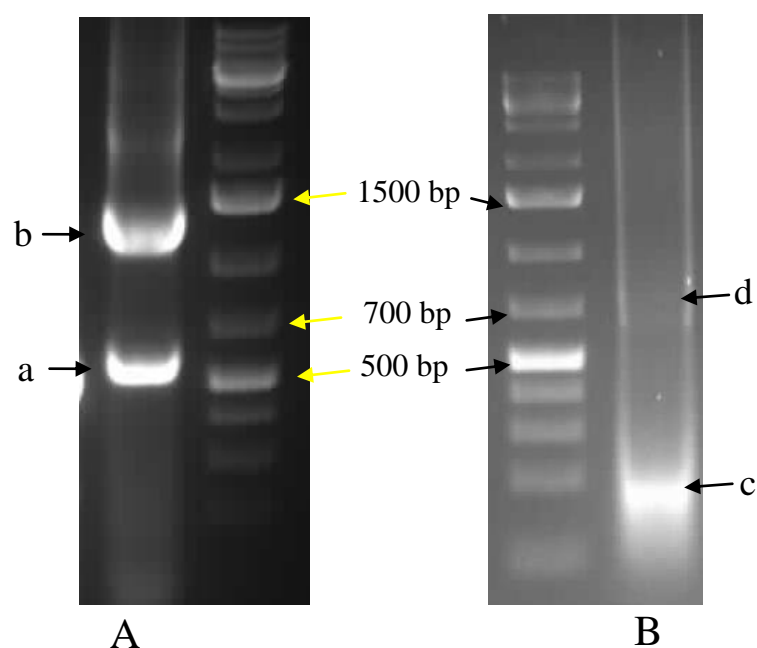
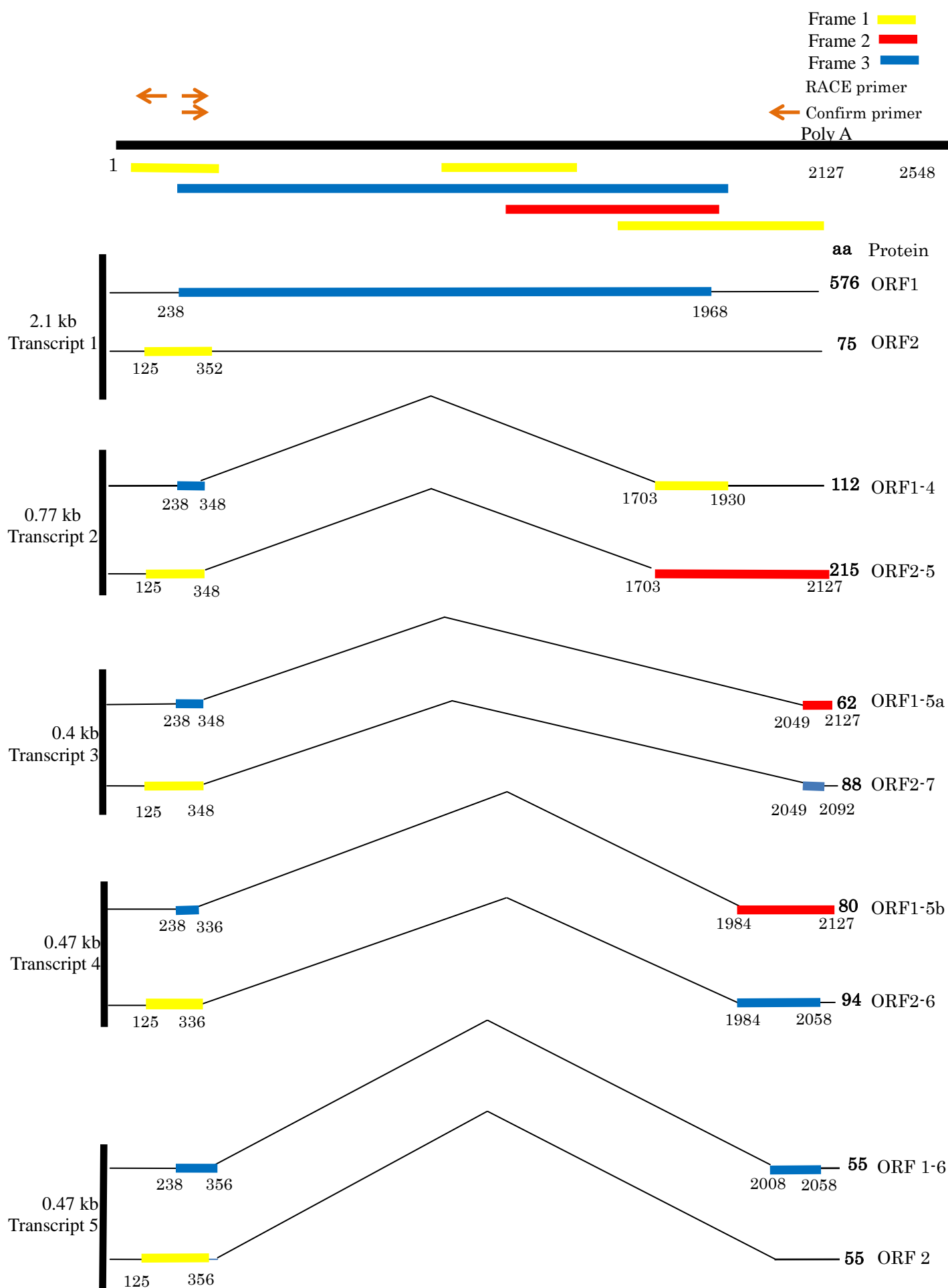
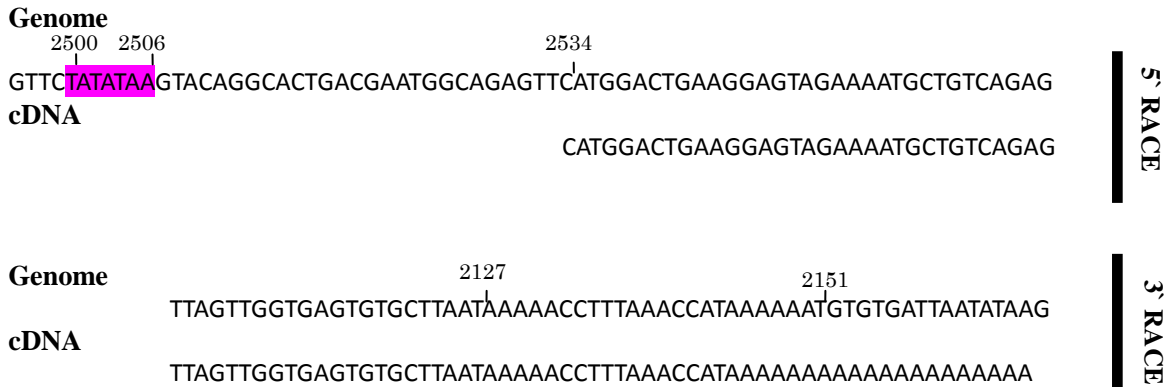


Figure 4.3.3. The PCR amplified products of RoTTV mRNA in various lengths. RT-PCR of RoTTV1 mRNA by using ORF2 and ORF3 primers (Table 2.12.1) was performed; (A) the band; a (580 bp) showed the shorter mRNA 0.8 kb and the band; b (1450 bp) showed the unspliced mRNA 1.7 kb. RT-PCR of RoTTV2 mRNA by using 3' RACE gene specific primers and the primers designed based on the upstream of poly A tail was performed; (B) the band; c (100-200 bp) showed the shorter mRNA making the smear and the band; d (700-900 bp) showed the larger mRNA.



B



C

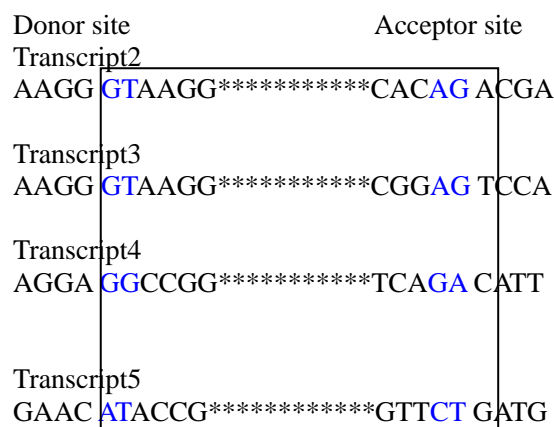


Figure 4.3.4. cDNA analysis of RoTTV2 (clone AS_WM1_Se_4). (A) Summary of the RoTTV2 transcriptional map. The genetic map of RoTTV2 is shown on the top. The initiation site and polyadenylation cleavage site are indicated with nucleotide numbers. . In addition, the primer sites of Race and conformation PCR were indicated as orange arrow. Nine predicted ORFs are shown below. The ORF1 and ORF2 proteins are encoded from the unspliced 2.1 kb mRNA by using O1 AUG (nt 238) and O2 AUG (nt 125), respectively. Other 0.77 kb, 0.4 kb, 0.47 kb, and 0.47 kb mRNAs are also predicted to initiate translation from O1 AUG (nt 238) and O2 AUG (nt 125). (B) Mapping of 5' and 3' end of RoTTV2 (clone AS_WM1_Se_4) transcripts. Mapping of 5' end of the RoTTV2 (clone AS_WM1_Se_4) transcripts and the genomic sequence with TATA box are given above. Also mapping of the 3' end of the RoTTV2 (AS_WM1_Se_4) transcripts with poly A (nt 2151) is shown. (C) Sequence of the donor and acceptor site in RoTTV2 (clone AS_WM1_Se_4). The consensus sequence of splice donor (SD) and splice acceptor (SA) sites in splicing site is shown. Transcript 3 and Transcript 4 do not followed the GT-AG splicing rule. Transcript3 shows GG-GA splicing and Transcript 4 shows the AT-CT splicing.

4.4. Characterisation of the spliced TTV mRNAs and Genomic Organization.

In the RoTTV1 clone AS_HL1_Li_1, the 1.7 kb mRNA (Transcript 1) is not spliced, and ORF2 was predicted to start at the first ATG codon (nt 125) in frame 3 and ORF1 was predicted to start at the second ATG codon (nt 259) in frame 2. Consequently, ORF2 would encode 116 aa in frame 3 and ORF1 would encode 342 aa in frame 2. The 0.8 kb mRNA (Transcript 2) had a single splicing event that bound nt 398 to nt 1289. The donor site at nt 398 was located 74 nt upstream of the stop codon of ORF2, creating the new ORFs: ORF2-3 (nt 125-398 and nt 1289-1575 in frame3) and ORF1-4 (nt 259-398 and 1289-1575 in frame2).

The donor and acceptor sites of the splicing event in RoTTV1 mRNA are shown in Fig. 4.3.2.C and follow the GT-AG splicing rule (Mount. 1982) and the intron consensus rule (Breathnach *et al.*, 1978). Finally, it was concluded that the coding region including the four ORFs is deduced to be nt 125-1708 and the noncoding region is located at nt 1708-2244 and nt 1-124 accounting for approximately 30% of RoTTV1 genome.

In the RoTTV2 clone, AS_WM1_Se_4, the 2.1 kb mRNA (Transcript 1) had no splicing, and ORF2 was predicted to start at the first ATG codon (nt 125) in frame 1 and ORF1 was predicted to start at the second ATG codon (nt 238) in frame 3. Therefore, ORF2 would encode 75 aa in frame 1 and ORF1 would to encode 576 aa in frame 3. The 0.77 kb mRNA (Transcript 2) had a single splicing event that bound nt 348 to nt 1703. The donor site of the splice at 348 was located at 4 nt upstream of the stop codon ORF2 creating other new ORFs: ORF 1-4 (nt 238-348 in frame 3 and nt 1703-1930 frame 1) and ORF2-5 (nt 125-348 in flame 1 and nt 1703-2127 in frame 2) encoding 112 aa and 215 aa respectively. The 0.4 kb mRNA (Transcript 3) had a single splicing event. Although the donor site of the splice was present at same position as that in the 0.77 kb mRNA, its acceptor site was located at nt 2049 suggesting the formation of other new ORFs: ORF1-5 (nt 238-348 in frame 3 and 2049-2127 in the frame 2) and ORF2-7 (nt 125-348 in flame 1 and 2049-2092 in frame 3) encoding 62 aa and 88 aa respectively. The 0.47 kb mRNA (Transcript 4) had a single splicing event that bound nt 336 to nt 1984. The donor site of the

splice at 336 was located at 16 nt upstream of the stop codon of ORF2 creating other new ORFs: ORF1-6 (nt 238-336 in frame 3 and 1984-2127 in frame 2), and ORF2-6 (nt 125-336 in frame 1 and nt 1984-2058 in frame 3) encoding 80 aa and 94 aa respectively. Another 0.47 kb mRNA (Transcript 5) had a single splicing event that bound nt 356 to nt 2008. The donor site of the splice at 356 was located 4 nt downstream of the stop codon of ORF2 and this mRNA encoded the complete ORF2 in frame 1 identical to 2.1 kb mRNA (data was not shown in Fig. 4.3.4.). The splicing event created a new ORF: ORF1-6: nt 238-356 and 2008-2058 in frame 3 encoding 55 aa.

The splice donor and acceptor sites in two RoTTV2 mRNAs followed the GT-AG splicing rule (Mount, 1982) (Fig. 4.3.2.C) but those of the two 0.47 kb mRNAs did not (Fig. 4.3.2.C). Two 0.47 kb cDNA clones and four 0.47 kb clones were isolated from PCR amplified products and these multiple clones support the presence of these new non-GT-AG splicing transcripts. It is known that the human erythrovirus B19 has a CT-AG splice (Brunstein *et al.*, 2000; Vashisht *et al.*, 2004; Kamada *et al.*, 2006) and RoTTV2 could also have these transcripts. Finally, it was concluded that the coding region including the four ORFs (ORF1-4) is deduced to be nt 125-2127 and the noncoding region was located at nt 2128-2571 and nt 1-124.

4.5. Predicted protein and localisation of RoTTV Fusion Protein

4.5.1. Prediction of RoTTV1 and RoTTV2 viral proteins

A high level of variability in ORF1 amino acid sequence, especially in RoTTV1 was observed in the RoTTV clones (as described in section 3.5). The ORF1 proteins of RoTTV1 and RoTTV2 have a conserved arginine-rich N-terminal region (Fig. 4.5.1.1.). In RoTTV2, the ORF1 protein has some rolling circle replication (RCR)-associated motifs identical to those found in other anelloviruses, CAV, and PCV capsid proteins (section 1.4.). In RoTTV2 ORF1, motif II (HXX) was located at aa 336-338 as HRH and motif III (YXXK) was located at aa 47-50 as YYRK. Motif I (FTL or FXTL) described in human TTV studies was not detected in RoTTV2. Accordingly, RoTTV1 and RoTTV2 showed a conserved arginine-rich N-terminal region but the RCR associated motifs were only observed in RoTTV2 and those of RoTTV1 might be less conserved or completely lost.

As described in other anelloviruses and CAV (Peng *et al.*, 2002; Peter *et al.*, 2002), RoTTV ORF2 contained a protein-tyrosine phosphate (PTP) motif (WX7HX3CXCX5H) at aa 14-34 for RoTTV1 and a protein-tyrosine phosphate (PTP) like motif was also observed at aa 15-35 for RoTTV2 but the amino acid sequence was FX7HX3CXCX5H. These motifs were preserved in the ORFs created by the spliced transcripts of RoTTV1 and RoTTV2.

In addition, RoTTV1 ORF2-3 showed a serine and lysine rich area at the C-terminus (Fig. 4.5.1.2.). TTV, TTMV, and CAV are known to have a similar motif in their ORF2-3 and this motif is similar to the phosphorylation site of the nonstructural protein hepatitis virus C (NS5A) (Asabe *et al.*, 2001).

RoTTV1 ORF1

MAW**R**FP**R**Q**R**AW**RR**KTW**R**PT**RRR**TY**RRR**TARR**RR**TRKW**RR**G**RR**

RoTTV2 ORF1

MAYFY**RR**TF**R**YP**RR**NNY**RRR**Y**RRRR**QYGT**RRR**TY**RRR**V**RR**PGKWR**YYR**KYS**RR**

Figure 4.5.1.1. Arginine-rich N-terminus in ORF1 of RoTTV1 (AS_HL1_Li_1) and RoTTV2 (clone AS_WM1_Se_4). Arginine residues are shown in red.

MNVDEDFIYKED**S**WLCMIRQTHELFCSCTSWTTHILKTLLDFNHPEWRGDSQDREPGEGK
 PGDQPDGAPTDEELLGVLEELENGDAGEGTSRPQTDSGSHTPQGWWPIEDQCNGNGQQ
 MENATKRPGGLQNLLKKLNTRFQPSKTKKKCKRKLKLKGKKRRYTTSKNTDTSSTDSS
 EYSTESSVFSSSDDDCWDNGPPLTPSQNMNWTQFISKLCSNVPVDPQLSPNKH

Figure 4.5.1.2. The entire amino acid sequence of RoTTV1 ORF2-3 (Clone HLWM). Serine residues are shown in red and basic amino acid, arginine and lysine, are underlined. Serine rich area with lysine and arginine residues was located in C-terminus of ORF2-3. WX7HX3CXCX5H motif was located in N-terminus of ORF2-3.

4.5.2. Subcellular localisation of RoTTV1 fusion protein

The identified transcripts of RoTTV1 were used as the basis for the fusion protein expression study. The coding sequences of RoTTV1 were amplified by PCR using restriction site containing primers (Table 2.13.1.). RoTTV1 complete plasmid (clone AS_HL1_Li_1) was used for amplifying ORF1 and ORF2 coding sequence and cDNA from RoTTV1 infected bone marrow (section 4.2. AS_HL1_Li_1) was used for amplifying the spliced coding sequence, ORF1-4 and ORF2-3. The amplified products by PCR were cloned to pGEM®-T easy then, subcloned in pEGFP-C1 (Clotech) and pcDNATM 3.1/ myc-His(-) A MCS (Invitrogen) plasmids using appropriate restriction enzymes. The recombinant plasmids were transfected into BHK-21 cells. RoTTV1 ORF fusion proteins were expressed and detected by fluorescence. For the pcDNATM 3.1/ myc-His (-) fusion transfected cells (section 2.13.4.), an anti-myc immune stain was required. The nuclei were counterstained by TO-PRO-3 iodide.

As shown in Fig. 4.5.2.1. (A, B) and Fig. 4.5.2.2. (A, B), the fusion proteins were expressed 24 h post-transfection, and two detection strategies (GFP or c-myc) showed similar results. The ORF1 protein localised the nucleus, especially to nucleolus-like structures (arrow heads) and the ORF2 protein predominantly localised to the cytoplasm with punctate granules around the nucleus (arrow). ORF1-4 was located in the cytoplasm and nucleus, especially in nucleolus like structures (arrow head). ORF2-3 was located in the cytoplasm with punctate granules (arrow) around the nucleus and also within the nucleus. RoTTV1 ORF 1, ORF2, ORF1-4 and ORF 2-3 correspond to human TTV genotype 1 ORF1, ORF2, ORF1-1, ORF2-2 and RoTTV protein expression patterns were similar to those of human TTV genotype 1 (Muller *et al.*, 2008).

At 48h post transfection, ORF1-4 transfected BHK-21 cells showed morphological changes such as fused cells Fig. 4.5.2.3.A or nuclear fragmentation like change Fig. 4.5.2.3.B. In apoptotic cells, nuclear fragmentation, formation of chromatin spheres with in the cytoplasm, and chromatin condensation are typically observed. Also, cellular swelling in apoptotic cells is found as secondary necrosis (Bishop *et al.*, 1993). It was predicted that ORF1-4 might have an

apoptosis inducing function similar to TAIP of human TTV or Apoptin of CAV (section 1.4.3.).

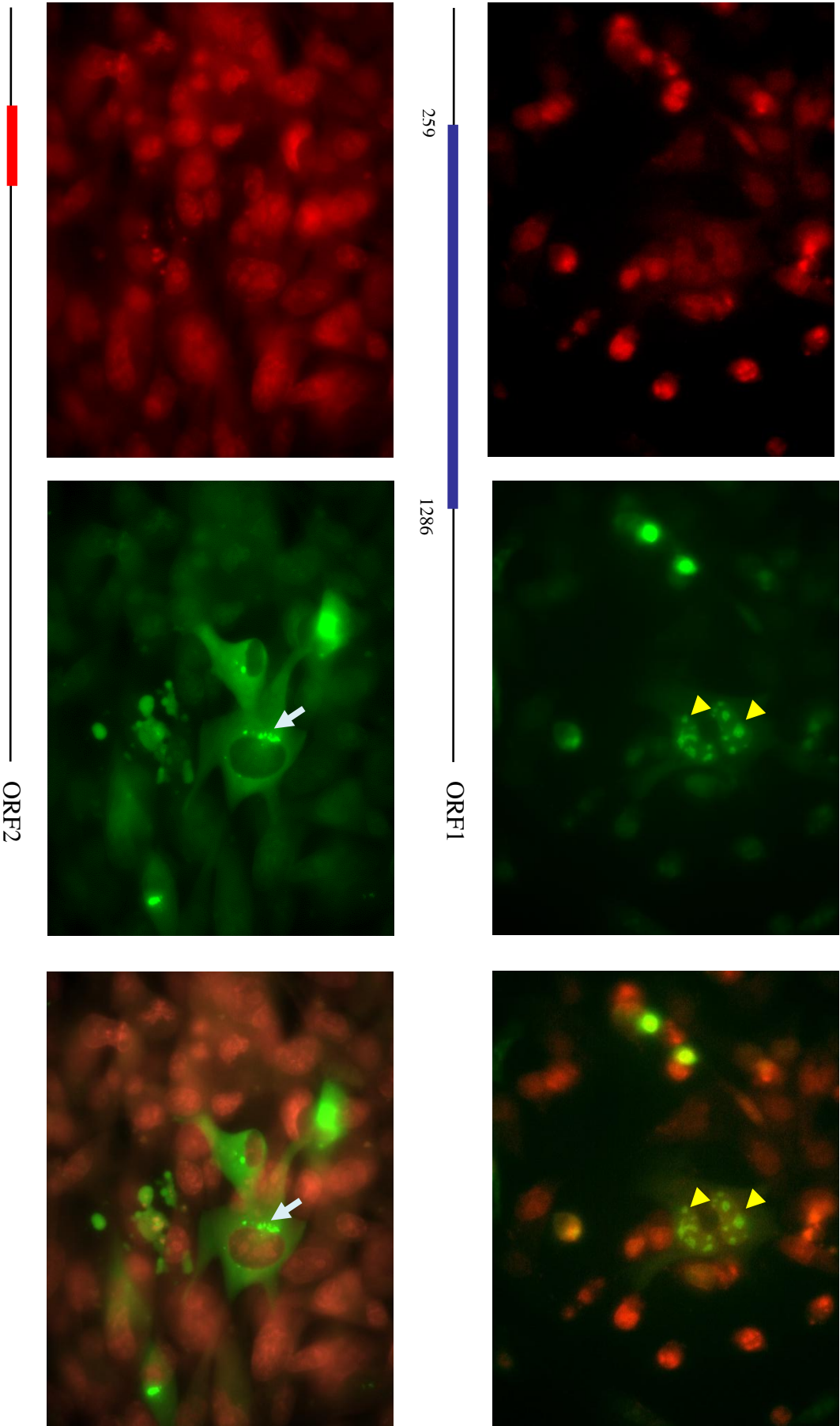


Figure 4.5.2.1.A. GFP/ORF fusion proteins were expressed in BHK-21 cells and visualized 24 h post transfection. ORF 1 protein localised to the nucleus, especially to nucleolus like structures (arrow heads) and ORF2 protein was predominantly localised to the cytoplasm with punctate granules around the nucleus (arrow). Red: TO-PRO-3, Green: GFP fusion protein.

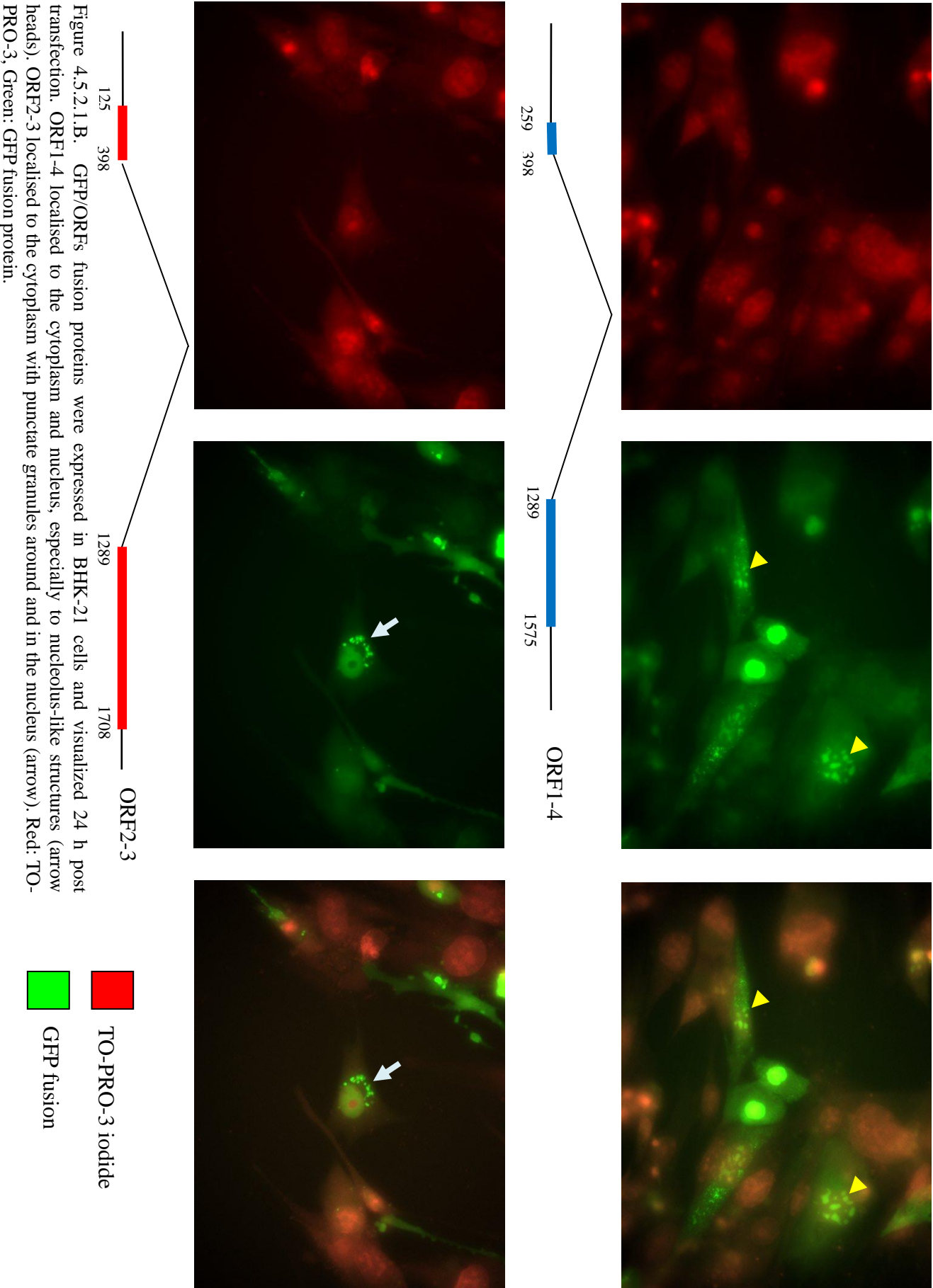


Figure 4.5.2.1.B. GFP/ORFs fusion proteins were expressed in BHK-21 cells and visualized 24 h post transfection. ORF1-4 localised to the cytoplasm and nucleus, especially to nucleolus-like structures (arrow heads). ORF2-3 localised to the cytoplasm with punctate granules around and in the nucleus (arrow). Red: TO-PRO-3, Green: GFP fusion protein.

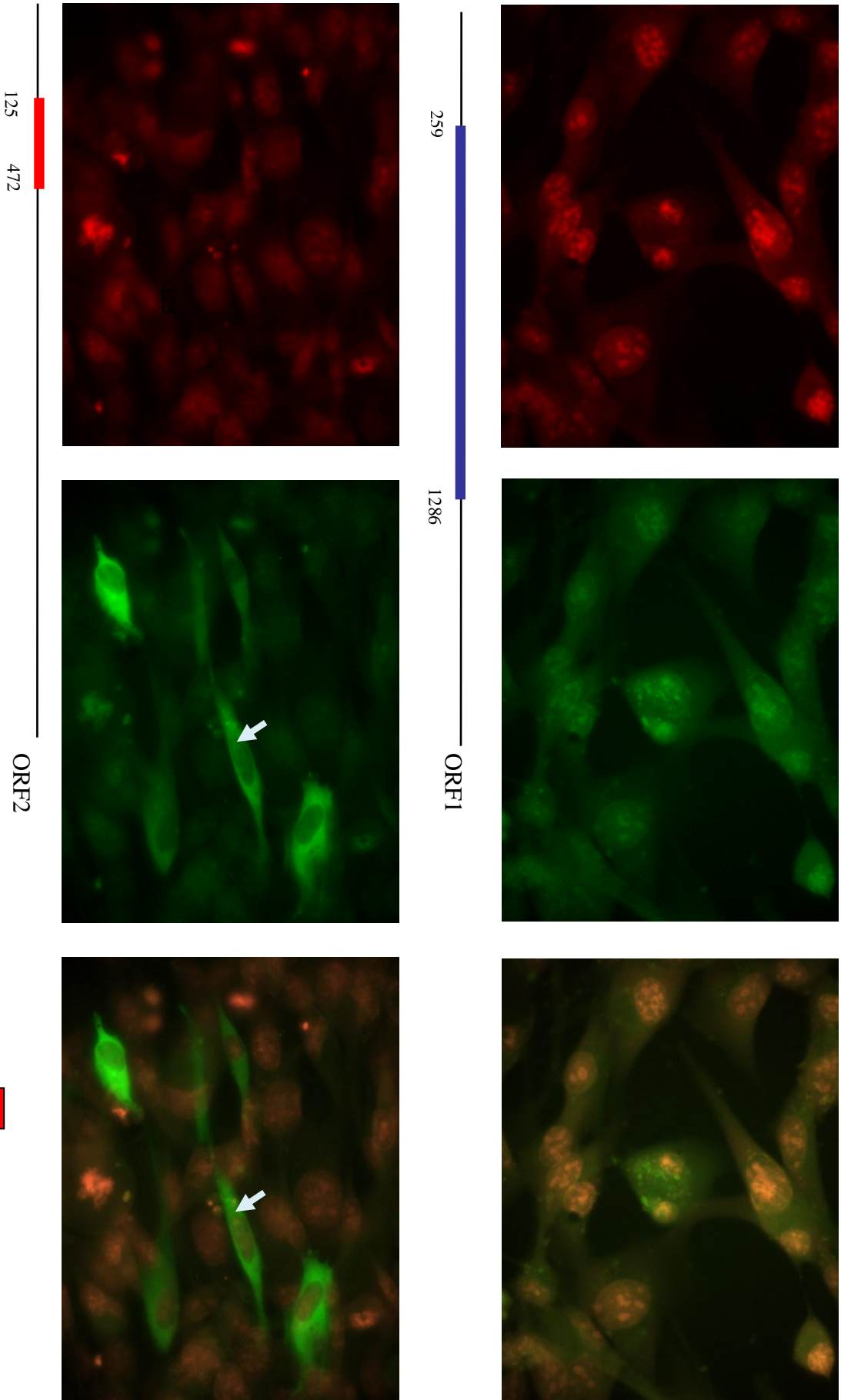
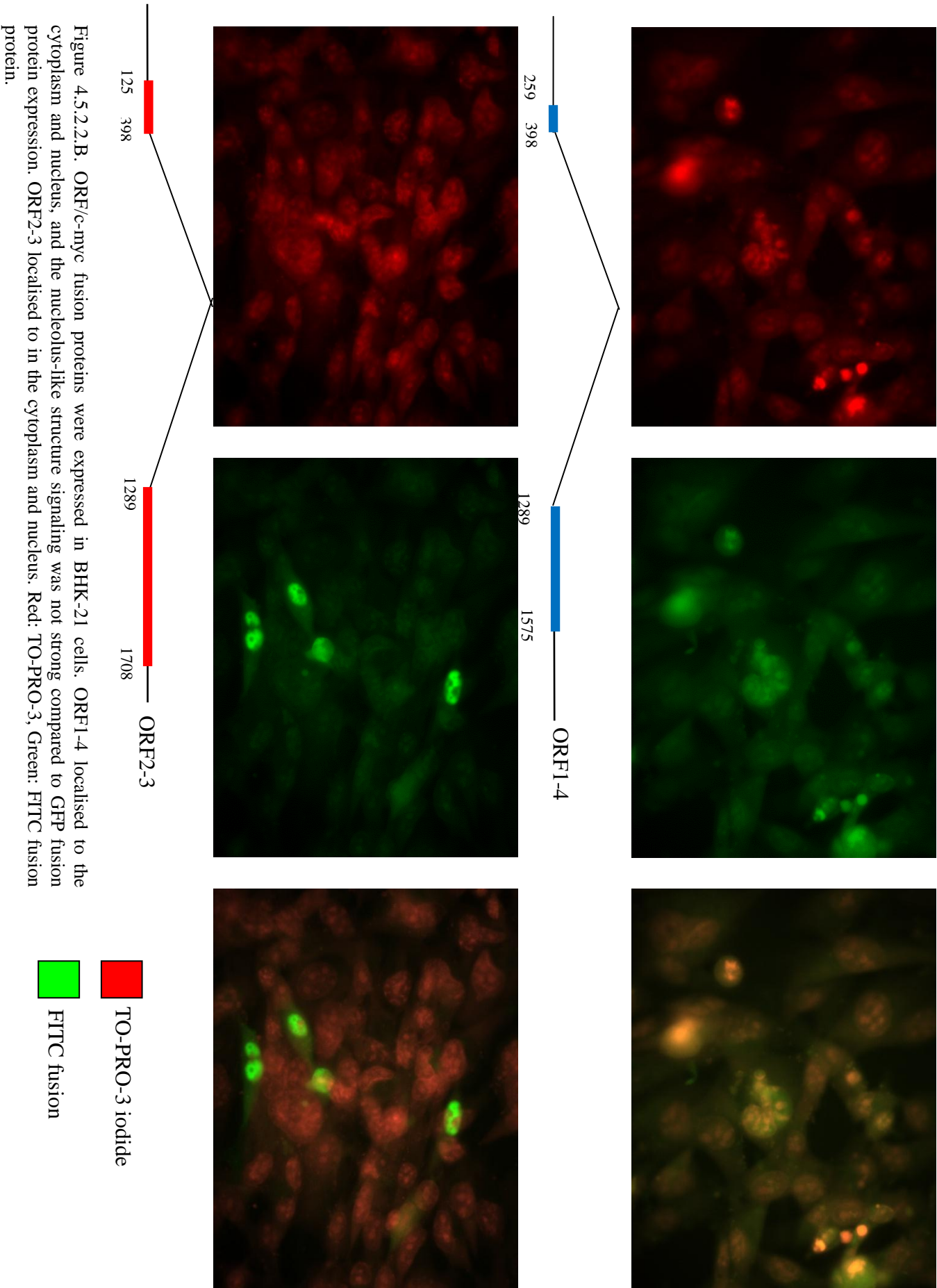
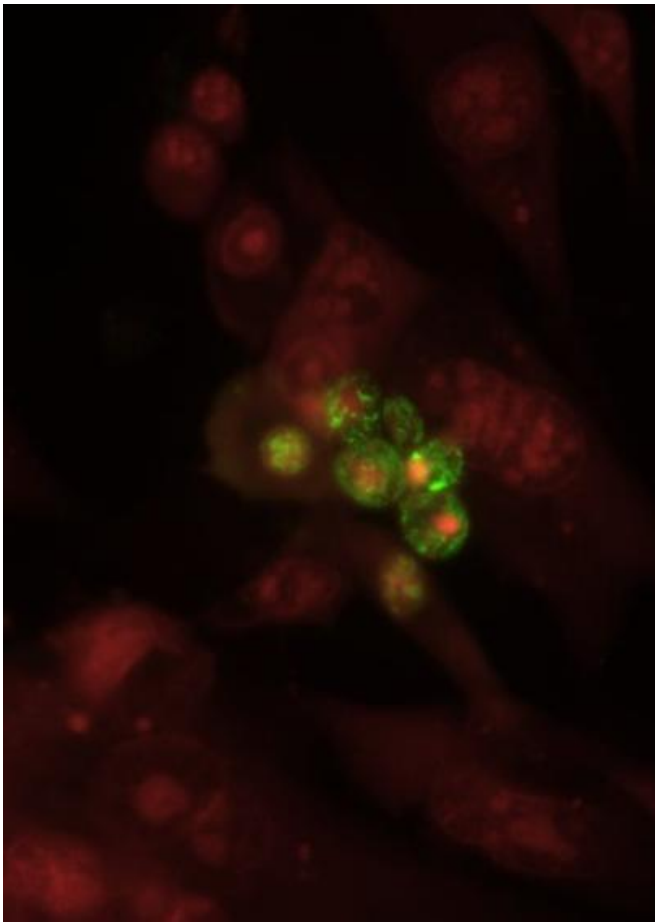


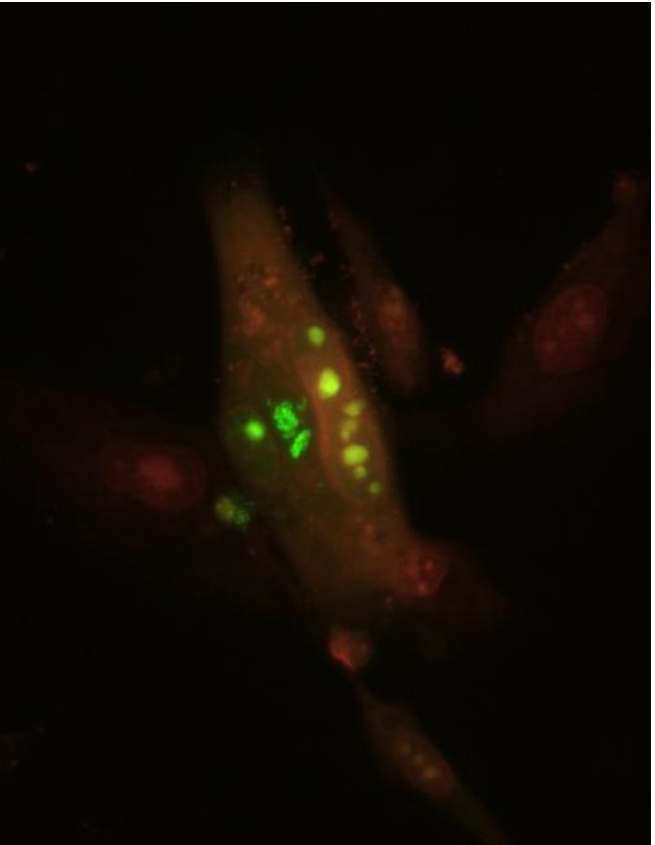
Figure 4.5.2.2.A. ORF/c-myc fusion proteins were expressed in BHK-21 cells and visualized 24 h post transfection. ORF 1 protein localised to the nucleus, and ORF2 protein predominantly localised to cytoplasm. The punctate granules around the nucleus were weaker than those seen with GFP expression (arrow). Red: TO-PRO-3, Green: FITC fusion protein.

B





(A)



(B)

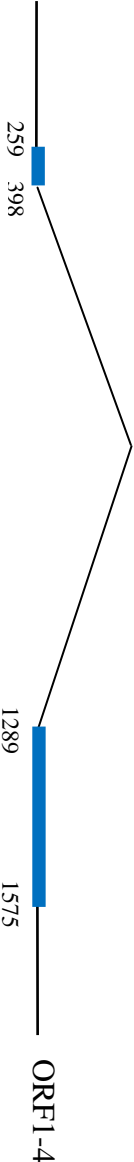



Figure 4.5.2.3. ORF1-4 and GFP fusion protein was expressed in BHK-21 cells and visualized 48 h post transfection. The BHK-21 cells showed nuclear fragmentation like change (A) or fused cells (B). Red: TO-PRO-3, Green: GFP fusion protein.

 TO-PRO-3 iodide

 GFP fusion

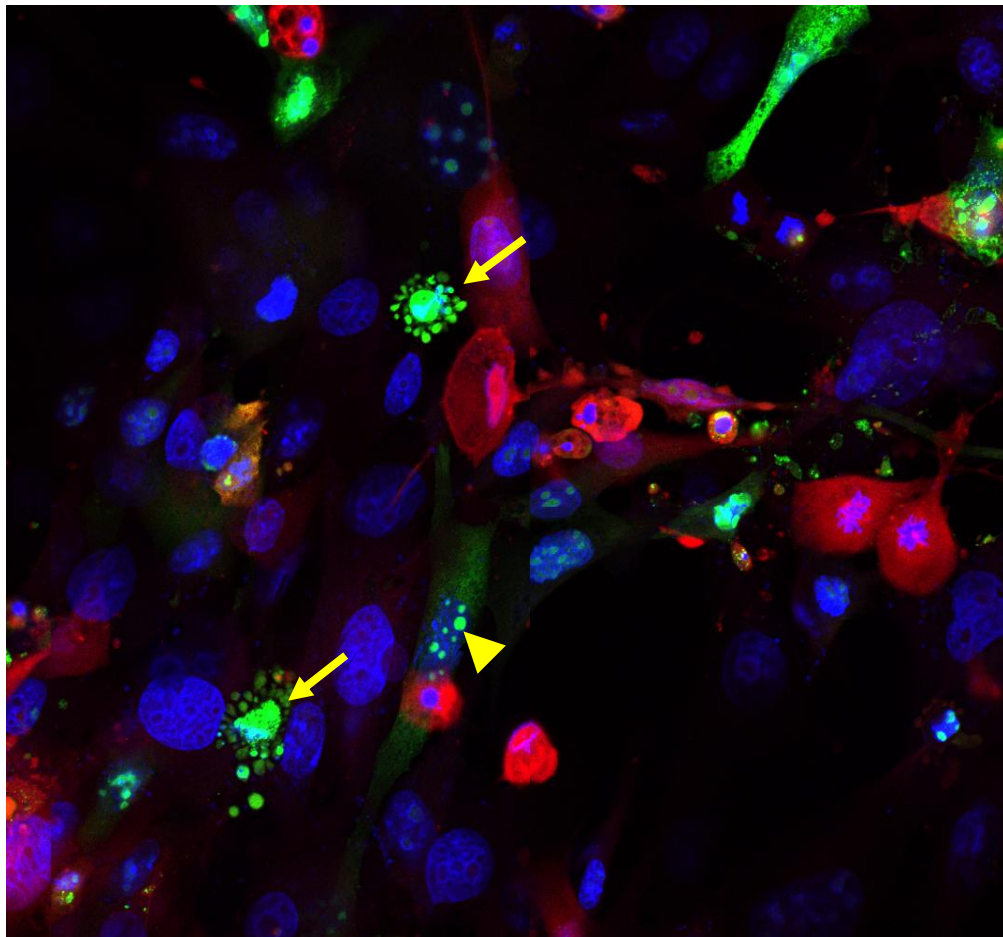
4.6. Apoptosis inducing capacity of RoTTV protein

4.6.1. Confocal microscopy of apoptosis stained cells

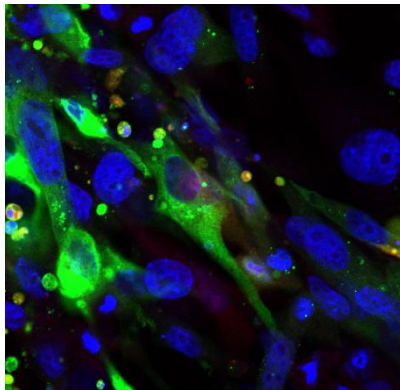
As shown in Fig. 4.5.2.3., the morphological changes in ORF1-4 transfected cells were similar to those of apoptotic cells. For studying the apoptosis inducing properties of ORF1-4, FLICA® 660 *in vitro* poly caspase detection (ImmunoChemistry Technology) was used to stain the apoptotic cells in ORF1-4 GFP fusion protein expressing BHK-21 cells. FLICA 660 poly caspase inhibitor probe contained the preferred binding sequence to bind all caspases (Val-Ala-Asp) and shows strong red fluorescence in apoptotic cells. The transfected cells were stained by FLICA and DAPI for the counter stain and the cells were analyzed by a Zeiss LSM710 confocal microscopy (Carl Zeiss, UK). The results are shown in Fig. 4.6.1.B. Following transfection of ORF1-4 GFP constructs, BHK-21 cells had many apoptotic cells but the fragmented cells which had a strong GFP expression were not stained by FLICA (Fig. 4.6.1.B (arrow)). This result was also observed for other RoTTV1 proteins (Fig.4.6.1. A, C). ORF1 was not studied as its presence could not be confirmed by western blot analysis (Data shown in section 4.7.)

4.6.2. FACS analysis of apoptosis stained cells/ confocal microscopy and cell counting

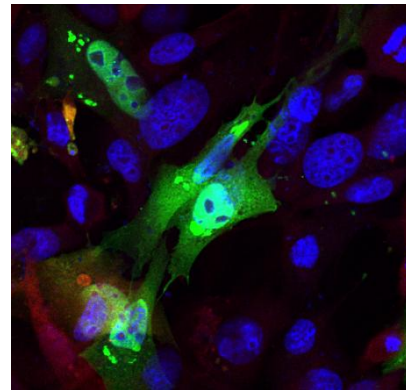
For counting the GFP expressed and FLICA stained cells, GFP fusion protein expressed BHK-21 cells were trypsinised and stained by FLICA, and these cells were analyzed by BD LSR Fortessa cell analyzer (BD Biosciences, USA). However, the staining conditions needed to be optimized for the FACS analysis and almost all cells were stained by FLICA (The data was not shown). Therefore, the stained and protein expressed cells were counted on the several images from section 4.6.1. The FLICA stained and GFP positive cells were manually counted by an experimenter in a blinded fashion. ORF1 was removed from this study because full length ORF 1 was found not to be expressed in BHK-21 cells (section 4.7.) The number of cells, natural apoptotic rate and fusion protein effects are shown in Table 4.6.2. The rate of virus fusion protein effects (FLICA and GFP double positive cells/total cell number) was not higher than naturally apoptotic rate (FLICA only positive cells/total cell number) of BHK-21 cells (Fig. 4.6.2.).



(B)



(A)



(C)

Figure. 4.6.1. FLICA staining in GFP and RoTTV1 ORF fusion protein. FLICA stained cells (apoptotic cells); red. GFP fusion protein; Green, DAPI (nucleus); Blue. (A) ORF2 GFP fusion protein, (B) ORF1-4 GFP fusion protein, (C) ORF2-3 GFP fusion protein. ORF1-4 expressing cells showed fragmentation (arrow) but FLICA staining was negative. Also, normal ORF1-4 expressing cells were FLICA negative (arrow head). This result was same as other ORFs; (A) ORF2 (C) ORF2-3.

	Blue* (DAPI positive) (FLICA-, GFP-)	Blue/Red[†] (DAPI/FLICA positive) (GFP-)	Blue/Green[‡] (DAPI/GFP positive) (FLICA-)	Blue/Red/Green[§] (DAPI/FLICA/GFP (positive)	Total
ORF2	66	8	42	4	120
ORF1-4	65	63	24	15	167
ORF2-3	95	22	18	8	143
GFP	29	9	62	10	110

Table 4.6.2. The number of FLICA positive and GFP expressing BHK-21 cells counted to identify RoTTV1 protein apoptosis inducing capacity. *Total number of cells. [†]The number of FLICA positive cells (apoptotic cells) but not to express GFP or fusion protein. [‡]GFP or GFP fusion protein expressed cells but FLICA was negative. [§]The number of GFP or GFP fusion protein and FLICA double positive cells.

ORF2 GFP fusion protein

Naturally apoptotic rate; $8/74=11\%$

Fusion protein effects; $4/46=8.6\%$

ORF1-4 GFP fusion protein

Naturally apoptotic rate; $63/128=49\%$

Fusion protein effects; $15/39=38.5\%$

ORF2-3 GFP fusion protein

Naturally apoptotic rate; $22/117=19\%$

Fusion protein effects; $8/26=30\%$

GFP fusion protein

Naturally apoptotic rate; $9/38=24\%$

Fusion protein effects; $10/72=14\%$

Figure. 4.6.2. Natural apoptotic rate and fusion protein effects. Naturally apoptotic rate was calculated following the formula; FLICA positive cells/total untransfected cell number (Blue+Blue/Red), and fusion protein effects was calculated following the formula; FLICA and GFP double positive cells/total GFP expressed cell number (Blue/Green+Blue/Red/Green). Fusion protein effects were smaller than naturally apoptotic rate in all RoTTV1 ORF fusion protein.

4.7. Confirmation of viral protein expression

To confirm the synthesis of the four RoTTV1 proteins and determine the molecular weight, RoTTV ORFs the N-termined were eGFP tag expressed in BHK-21 cells and protein expression at 24 h post transfection was verified by western blot analysis. Anti-GFP antibody (sc-8334, Santa Cruz) was used for the blotting and the result is shown in Fig. 4.7.1.1. GFP was detected in a band at approximately 26.9 kDa and fusion proteins were detected in a band size at approximately 50 kDa and 38 kDa (predicted size 40 kDa) for ORF2, approximately 50 kDa (predicted size 42.6 kDa) for ORF1-4 and approximately 65 kDa and 38 kDa (predicted size 52.5 kDa) for ORF2-3. ORF2 and ORF2-3 showed two bands and the lower bands were similar in size to each other. However, by analysing Splicing Site Prediction (www.fruitfly.org/seq_tools/splice.html) and PeptideCutter (web.expasy.org/peptide_cutter/), it was not clear how the lower bands of ORF2 and ORF2-3 were generated. The actual sizes of the proteins were slightly higher than predicted and this may indicate that protein modifications such as glycosylation, phosphorylation, and acetylation have occurred. Although, the ORF1 protein was detectable by both anti c-myc immune stain and GFP detection in fixed cells, ORF1 protein was not detectable by Western blot analysis.

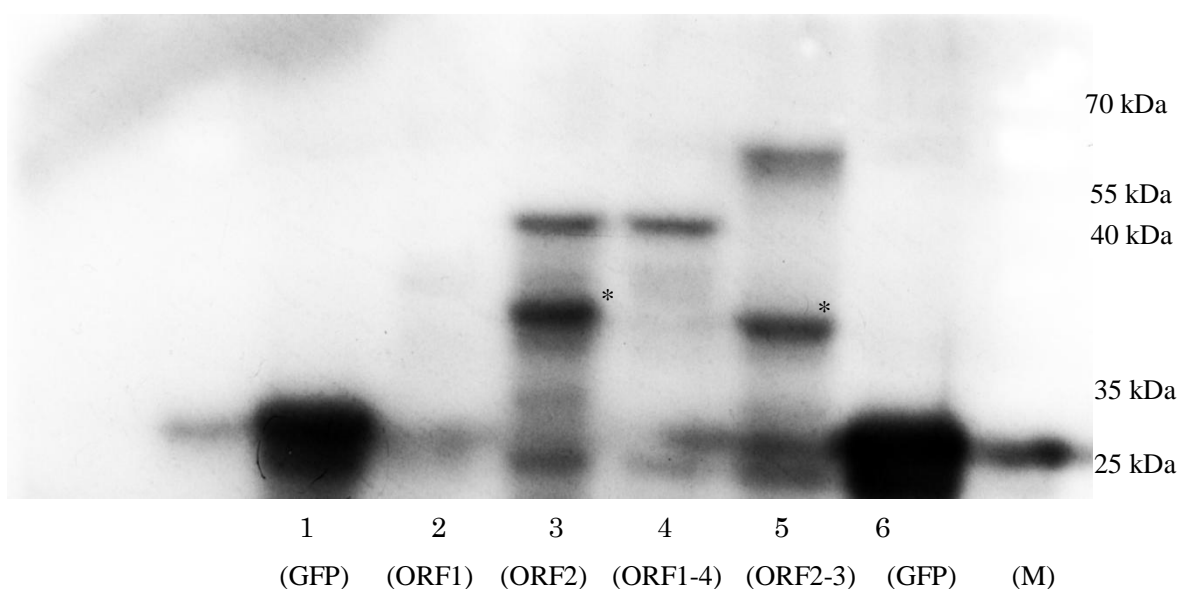


Figure 4.7.1.1. Detection of recombinant RoTTV1 proteins by western blot analysis with anti GFP antibody. (M) PageRuler (Thermo scientific). (1 and 6) positive control (pGFP C-terminus), (2) pGFP ORF1 fusion protein, (3) pGFP ORF2 fusion protein, (4) pGFP ORF1-3 fusion protein, (5) pGFP ORF2-3 fusion protein. *ORF2 and ORF2-3 showed similar sized lower bands.

4.8. Discussion

4.8.1. RoTTV1 RNA distribution

The present study showed that RoTTV1 RNA was predominantly distributed in the bone marrow and lung tissue and indicated that these two tissues are the replication sites of RoTTV1. Human TTV replication site has been studied in TTV infected patients (Okamoto *et al.*, 2001; Kikuchi *et al.*, 2000; Lopez-Alcorocho *et al.*, 2000) and TTV DNA and RNA have been found in various tissues including liver, bone marrow, lung, spleen, pancreas, kidney, lymph node, skeletal muscle, and thyroid gland although the TTV viral load was different among the individuals and higher in bone marrow, lung, spleen, and liver (Okamoto *et al.*, 2001). However, it has also been suggested that TTV replicates in cells of the immune system and the immune cells may be the carrier of replicating virus. Detection of viral RNA or double-stranded intermediate of TTV throughout tissues may be due to the presence of immune cells (Takahashi *et al.*, 2002). When TTV was discovered it was thought to be a hepatitis virus (section 1.5.2.1.) but other reports have disputed this hypothesis (Hijikata *et al.*, 1999; Hsieh *et al.*, 1999; Nakano *et al.*, 1999; Naoumov *et al.*, 1998; Niel *et al.*, 1999; Prati *et al.*, 1999; Viazov *et al.*, 1998; Yamamoto *et al.*, 1998; Nobili *et al.*, 2005). In the present study, the liver was not the main replication site compared to the other organs and this finding is supported by recent human TTV studies. Also, bone marrow cells including haematopoietic cells (Okamoto *et al.*, 2000; Takahashi *et al.*, 2002; Mariscal *et al.*, 2002; Maggi *et al.*, 2001; Lopez-Alcorocho *et al.*, 2000; Mariscal *et al.*, 2002; Zhong *et al.*, 2002) and cells in respiratory tract (Bando *et al.*, 2001; Maggi *et al.*, 2003a; Okamoto *et al.*, 2001) have been better studied in recent years (Aramouni *et al.*, 2003) and human TTV RNA levels were highest in these tissues. Also, in the present study, RoTTV1 RNA titre of bone marrow was 1000 fold higher than that of lung, and human TTV was suggested to infect by using faecal-oral route (section 1.3.2.2.). Therefore, we conclude that RoTTV1 could replicate in lung but continuous replication may occur in the bone marrow cells.

The replication site may be related to the TTV associated disease. In respiratory disease, it has been shown that TTV replication and high viral titer were observed in the pulmonary epithelium

of 1-24-month-old children with acute respiratory disease, and group 4 TTV was frequently observed in bronchopneumonia patients (Bando *et al.* 2001; Maggi *et al.* 2003a; Okamoto *et al.*, 2001; Chung *et al.*, 2007). Also, in haematologic disease, aplastic anemic and thrombocytopenia were also suggested as human TTV related disease (Kikuchi *et al.*, 2001; Tokita *et al.*, 2001). Therefore, RoTTV1 infected rodents may show respiratory disease or haematologic disease in associated with TTV infection.

The RoTTV RNA expression pattern may depend on the virus genotype. It was found that human TTV from PBMC and plasma showed different genotypes in the same individual (Okamoto *et al.*, 1999; Okamoto *et al.*, 2000). Thus, it was concluded that RoTTV1 would replicate in lung and bone marrow cells but it may not be same as RoTTV2 or other human and animal TTVs.

Bone marrow is also a predicted replication site of human and animal TTVs. TTV is small single-stranded DNA virus and small DNA viruses tend to have the highest dependence on the host cell replication system for virus replication. TTV is also predicted not to encode a DNA polymerase based on the genomic analysis (Kakkola *et al.*, 2007). Therefore, TTVs have to infect highly replicating cells which are in S-phase and the bone marrow may be the main replication site of RoTTV1 for this reason.

4.8.2. Transcriptional profile and species specific structure of RoTTV transcript

The results gained in the present study indicated that at least two RoTTV1 mRNAs of 1.7 kb and 0.8 kb in size are transcribed in lung and bone marrow of a RoTTV1 infected wild mice, and at least five RoTTV2 mRNA of 2.1 kb, 0.77 kb, 0.4 kb, 0.47 kb and 0.47 kb are transcribed in RoTTV2 complete genome transfected BHK-21 cells. Their transcriptional profile was similar to those of human and porcine TTVs. Both mRNAs from RoTTV1 and all five mRNAs from RoTTV2 are transcribed from a common internal promoter in their genomes such as TATA-box (ATATAA in RoTTV1 and TATATAA in RoTTV2) at nt 2146-2150 and nt 2500-2506 respectively. The extreme 5' end of RoTTV1 mRNA was located at the position nt 2174 which is

24 nt downstream of the putative promoter (Fig. 4.3.2.B). Also, the extreme 5' end of RoTTV2 mRNA was located at the position nt 2534 which is 28 nt downstream of putative promoter (Fig. 4.3.4.B). These AT-rich promoter regions are located about 25 nt upstream of the mRNA start points in cellular and viral promoter and they are considered to bind with RNA polymerase for the initiation of transcripts (Corden *et al.*, 1980). Furthermore, all sequences from 3' RACE contained poly A starting immediately downstream of the polyadenylation signal (AATAAA) located at nt 1697-1702 in RoTTV1 and nt 2125-2130 in RoTTV2. Therefore, RoTTV1 and RoTTV2 would use the common internal promoter and poly A signal and shared common 5' and 3' ends. This is in agreement with human and porcine TTVs studies (Okamoto *et al.*, 2000).

In RoTTV1, the unspliced transcript was 1.7 kb in size and encodes ORF1 (nt 259-1286) and ORF2 (nt 125-472). The shorter transcript which had a splicing event at nt 398-1289 creates the new ORFs ORF1-4 and ORF2-3. These ORFs used the same ATG codons at 259 and 125 as ORF1 and ORF2 respectively. Also, the splicing event follows the GT-AG rule do as other TTVs and CAV (Mount 1982; Kamada *et al.*, 2006; Mueller *et al.*, 2008. Fig. 4.3.2.C). On the other hand, RoTTV2 showed five transcripts, 2.1 kb, 0.77 kb, 0.4 kb, 0.47 kb and 0.47 kb. It was also speculated that the unspliced 2.1 kb transcript encoded ORF1 (nt 238-1986) and ORF2 (nt 125-352) and other spliced transcripts would use the first methionine (ATG) of ORF1 and ORF2. Four splicing events were found in RoTTV2 and two of them were in accordance with GT-AG splicing pattern (Okamoto *et al.*, 2000) but other two showed GG-GA and AT-CT splicing patterns. Human TTV, parvovirus, CAV and other small DNA viruses all show the common GT-AG splicing pattern (Okamoto *et al.*, 2000; Vashisht *et al.*, 2004; Kamada *et al.*, 2006; Muller *et al.*, 2008) but erythrovirus B19 has been reported to have a CT-AG splice (Brunstein *et al.*, 2000) and was predicted to use a novel splicing pattern or pathway. Several GG-GA and GT-AG spliced transcripts were isolated from RoTTV2 RNA and they would utilise an uncharacterised splicing mechanism.

4.8.3. Common splicing site of RoTTV1 and RoTTV2

A common splicing site between RoTTV1 and RoTTV2 was found. For human TTV transcripts, all identified human TTVs transcripts have a splicing event (nt 182-284 in genotype 6 (Qiu *et al.*, 2005) and nt 185-277 in genotype1a (Kamahora *et al.*, 2000) which does not affect ORFs (Okamoto *et al.*, 2000; Qiu *et al.*, 2005; Muller *et al.*, 2008). However, RoTTV1 and RoTTV2 do not have this first splicing event in this study and this first splicing event also has not been observed in porcine TTV and CAV transcriptional studies (Kamada *et al.*, 2006; Martinez-Guino *et al.*, 2011). Animal TTV transcriptional profile has not been well-studied but it is possible that animal TTVs do not have first splice. The other common splicing event is found in the middle of ORF2 with overlapping part of ORF1 in human and porcine TTVs creating new ORFs (Okamoto *et al.*, 2000; Martinez-Guino *et al.*, 2011). This is also observed in RoTTV1 and RoTTV2 creating two new ORFs in RoTTV1 (ORF1-4 and ORF2-3) and seven new ORFs (ORF1-4, ORF2-5, ORF1-5a, ORF2-7, ORF1-5b, ORF2-6, ORF1-6) in RoTTV2.

4.8.4. ORF proteins of RoTTV1, predicted function, and protein localisation

ORF1; ORF1 is predicted to encode the capsid protein and Rep protein of TTV (Takahashi *et al.*, 1998; Bendinelli *et al.* 2001; Takahashi *et al.* 1998). However, no rolling-circle replication (RCR) motifs (motif 1; FTL, motif 2; HXH or HXQ, and motif 3; YXXK) were observed in RoTTV1. These motifs were observed in human and animal TTVs but the appearance patterns are variable in different species and clones of TTV and some motifs were lost or less conserved (Muller *et al.*, 2008; Martinez-Guino *et al.*, 2011). RCR motifs were not observed in RoTTV1 but motif II and motif III were found in RoTTV2 ORF1. By using these motifs, it has been deduced that ORF1 works as a replicase (Muller *et al.*, 2008).

In this study, the ORF1 fusion protein localised to the nucleus, especially in nucleolus like structures. This is in agreement with the recent human and porcine TTV reports (Muller *et al.*, 2008; Martinez-Guino *et al.*, 2011). There are two reports describing human TTV ORF1 expression in cell culture (Qiu *et al.*, 2005; Muller *et al.*, 2008), and one report indicated ORF1

was expressed in cytoplasm (Qiu *et al.*, 2005) and the other report showed ORF1 in nucleus (Muller *et al.*, 2008). ORF1 is suspected to be the capsid protein and also related to replication by using a rep-motif. Also, human TTVs and RoTTV have an Arg-rich region in the N-terminus of ORF1 and it is known to be most conserved region in ORF1 (Fig. 4.5.1.1.) (Bendinelli *et al.*, 2001; Takahashi *et al.*, 1998). This region showed homology to the nucleolar localisation signals (NuLS) of oncogene MEQ of a herpesvirus (Marek's disease virus; MDV) (Liu *et al.*, 1997) and adenovirus protein V (Matthews *et al.*, 2001) to induce the nucleolar transport (reviewed by Hiscox 2002). Furthermore, in human TTV a similar localisation pattern of ORF1 was confirmed as nucleolar (Muller *et al.*, 2008). Accordingly, RoTTV1 ORF1 expression in the nucleus may indicate it plays an important role in synthesising DNA. However, the full length ORF1 coding sequence was difficult to amplify by PCR (required 35 cycles in both rounds of nested PCR) and ORF1 GFP fusion proteins were not detected by western blot (Fig. 4.7.1.). This result is unexpected for a predicted capsid protein, but the same result was also reported in human and porcine TTV studies (Qiu *et al.*, 2005; Martinez-Guino *et al.*, 2011). One suggestion is the ORF1 coding sequence is unstable or low copy number. In this study, the unspliced RoTTV1 ORF1 mRNA was not amplified from bone marrow cDNA but could be found in the lung. However, bone marrow showed higher cDNA copy number and it is believed to be main replication site. Thus, TTV copy number in bone marrow should be high enough to detect virus ORF1 mRNA. In human TTV studies, premature stop codons in the middle of TTV ORF1 were observed suggesting the production of defective TTV genomes which also could indicate that ORF1 is unnecessary (Erker *et al.*, 1999; Luo *et al.*, 2000; Khudyakov *et al.*, 2000; Pollicino *et al.*, 2003; Jelcic *et al.*, 2004). RoTTV1 is likely to replicate in bone marrow but may not produce unspliced RNA transcripts. This also suggested that the full length ORF1 might not be necessary for TTV replication. In the porcine TTV study, the unspliced transcript has not been found yet and it is indicted that some animal TTV might not to have ORF1 (Martinez-Guino *et al.*, 2011).

Even when ORF1 coding sequence is predicted, it has also been difficult to detect the ORF1 protein (Qiu *et al.*, 2005; Martinez-Guino *et al.*, 2011). ORF1 was not detected in the first human

TTV protein studies (Qiu *et al.*, 2005) and porcine TTV studies (Martinez-Guino *et al.*, 2011). In the human study, it was concluded that ORF1 expression level was too low to detect it by using Western blot regardless of being a predicted structural protein (Qiu *et al.*, 2005). In a porcine TTV study, it was concluded that ORF1 protein might be expressed in the cell culture but the protein was unstable and degraded rapidly (Martinez-Guino *et al.*, 2011). In the present study, the samples for western blot were collected by using PBS containing proteinase inhibitor. However, ORF1 protein was not detected by western blot (Fig. 4.7.1.). One human TTV report succeeded in detection of ORF1 protein by using pCMV-HA plasmid and it was concluded that genogroup 1 (clone P/1C1) promoter may be more tightly regulated than the heterologous CMV promoter and therefore the use of different plasmids may allow the detection of ORF1 by Western blot (Muller *et al.*, 2008). Accordingly, the result might change, when other plasmids which have other promoter system are used in the study. The alternative may be that full-length ORF1 is never expressed.

ORF1-4; In this study, ORF1-4 fusion protein was also expressed in nucleus especially in nucleoli like structures and in the cytoplasm. Expression in the nucleoli like structures was more frequently observed than for ORF1. Also, Arg-rich region which has NuLS like sequence in N-terminus of ORF1 was conserved in ORF1-4 (see ORF1 above) and these nucleoli-like structures in the nucleus were confirmed as the nucleoli in human TTV study (Muller *et al.*, 2008). Some DNA and RNA virus proteins tend to be observed in nucleoli; for example, the replication complex of Borna virus for the replication and transcription (Pyper *et al.*, 1993), Rev and Tat protein of HIV-1 for RNA processing (Michienzi *et al.*, 2000), IVa2 and V of adenovirus for the delivery of viral DNA to the host cell during the infection (Matthew *et al.*, 1998). These viral proteins are considered to be related to cellular transcription (Liu *et al.*, 1997), virus transcription (Matthews *et al.*, 1998), virus translation (Hiscox *et al.*, 2001) or cell division (Wurm *et al.*, 2001). Therefore, ORF1-4 would be predicted to have an important role of virus infectious cycle similar to other viruses.

ORF1-4 transfected cells showed the morphological changes such as nuclear fragmentation and formation of the chromatin sphere 48 hours post infection and the intensity of GFP expression was decreased compared to 24 hours post infection. These morphological changes are typical of apoptosis (Bishop *et al.*, 1993) and human TTV protein is known to have a protein associated with the induction of apoptosis, TTV apoptosis inducing protein (TAIP) (Kooistra *et al.*, 2004). CAV unspliced mRNA encodes VP2 and VP3 proteins and they have an apoptosis inducing activity (Zhuang *et al.*, 1995; Noteborn and van der Eb. 1999; Noteborn *et al.*, 2005) and human TTV ORF3 showed the homology to CAV VP3 (Kooistra *et al.*, 2004). CAV VP3 showed a high level of apoptosis inducing capacity in tumours regardless of the tumour origin, but TTV TAIP induced apoptosis to hepatocellular carcinoma (HCC) preferentially and the apoptosis inducing capacity was weak in non-HCC cell lines (Kooistra *et al.*, 2004). Accordingly, human TTV would use a different apoptosis inducing mechanism from that of CAV. In this study, the conserved hydrophobic region (L or (I), S or (A), XIXIXLXL) of apoptin and TAIP was not observed in the RoTTV1 amino acid sequence.

To confirm the apoptosis inducing capacity, FLICA apoptosis detection kit was used for apoptosis staining. However, the cells which showed the morphological changes, were negative for FLICA and the apoptotic rate of GFP fusion protein expressed cells was lower than the natural apoptotic rate (section 4.6.2.). FLICA detects activated caspases apoptotic cells by using the probe and all apoptotic cells should activate at least one caspase (Liang *et al.*, 2001). Thus, we concluded that RoTTV1 protein does not have apoptosis inducing capacity and the morphologic changes in ORF1-4 transfected BHK-21 cells were thought to be caused by necrosis or other viral protein effects.

ORF2; ORF2 is assumed to code for a nonstructural protein involved in viral replication (Okamoto *et al.*, 1998. Reviewed in Bendinelli *et al.*, 2001), and the ORF2 protein expression pattern supported this hypothesis. ORF2 was predominantly expressed in the cytoplasm, and this is in agreement with previous human TTV studies. Also, in the present study, punctate granules

were observed around the nucleus (Fig. 4.5.2.1.A.). Some virus proteins localised to the cytoplasm which produce punctate granules and two types of granules have been reported; Golgi apparatus shown in herpes simplex virus (Nozawa *et al.*, 2003) or endoplasmic reticulum (ER) shown in Dengue virus (Miller *et al.*, 2006). The granules distributed in Golgi apparatus were shown around nucleus (Nozawa *et al.*, 2003) and the granules distributed in ER were found in almost entirely in the cytoplasm (Miller *et al.*, 2006). ORF2 granules were similar to those of Golgi apparatus and should be confirmed by Golgi-58K staining. If TTV ORF2 protein was located in Golgi apparatus the protein may be targeting the Golgi membrane or intracellular trafficking pathway of progeny capsids.

ORF2 also showed the common WX7HX3CXCX5H motif in RoTTV1 (Fig. 4.5.1.2.), and CAV VP2 is known to have same motif and it has protein-tyrosine phosphatase (PTPase) activity which regulates the cellular and/or viral protein during infection (Peter *et al.*, 2002). Therefore, RoTTV ORF2 could have a similar function to that of CAV. This motif was also conserved in ORF2-3 and is presumed to have a similar function (Fig. 4.5.1.2.).

In the western blotting analysis, ORF2 showed two bands of 50 kDa and 38 kDa in size (predicted size 40 kDa) and the lower band size of ORF2 was similar to the lower band of ORF2-3. In the human study, a stop codon followed by an ATG were located in the middle of ORF2 and it was divided into two smaller protein coding areas, ORF2a and ORF2b in some clones (Tanaka *et al.*, 2000). However, RoTTV has no stop codon in ORF2 and it is considered that the smaller bands were result of the post-translation cleavage or splice variants of mRNA (Reviewed in Stryer, 2001).

ORF2-3; Compared to ORF2, ORF2-3 localised to the nucleus and cytoplasm and it indicated that the structure or amino acid sequence encoded by the ORF3 region would contain the nuclear localisation signals. The N-terminus region of the ORF2-3 amino acid sequence was identical to that of ORF2 and it contained the WX7HX3CXCX5H motif and is predicted to have a protein -

tyrosine phosphatase (PTPase) activity that regulates the cellular and/or viral proteins during infection (Peter *et al.*, 2002) (see ORF2 mentioned above).

ORF2-3 showed a serine-, arginine-, and lysine- rich region in the 100 aa at the C-terminus (Fig. 4.5.1.). This region is similar to human TTV, TTMV and CAV (Asabe *et al.*, 2001) and the predicted protein could be phosphorylated similar to hepatitis C virus (HCV) nonstructural protein 5A (NS5A) (Asabe *et al.*, 2001). This phosphorylated human TTV ORF3 is predicted to be involved in the virus replication cycle and this is also observed in other virus infections such as vesicular stomatitis virus (Barick *et al.*, 1992; Spadafora *et al.*, 1996; Hwang *et al.*, 1999), the origin binding protein of the herpes simplex virus (Isler *et al.*, 2001), the NS1 protein of the minute virus of mice (Nuesch *et al.*, 1998), and the capsid protein of hepadnaviruses (Yu *et al.*, 1994). Therefore, RoTTV1 ORF2-3 could be phosphorylated in the same way as other viruses and work in the virus replication phase.

ORF2-3 was also expressed not only in cytoplasm but also in the nucleus indicating that the region encoded by ORF3 contained the nuclear localisation signal (NLSs) like "pat4" which consists of a continuous stretch of four basic amino acid (arginine and lysine) (Robbins *et al.*, 1991; Hiscox *et al.*, 2002). This supports ORF2-3 localisation in nucleus.

Western blot analysis showed two bands of ORF2-3 proteins and the lower band was a similar size to ORF2 lower band (see ORF2) suggesting that ORF2-3 may also be spliced or cleaved in a similar manner to ORF2 (see above). In this study, almost all proteins detected by western blot showed larger products compared to the predicted protein sizes and this was also shown in a human TTV study (Muller *et al.*, 2008). There are several factors that may influence the product size. The expressed fusion proteins may be targeted for protein modification such as glycosylation, phosphorylation, and acetylation, and it would give products of a higher molecular weight than the predicted bands (reviewed in Stryer, 2001). Furthermore, the proteins may be aggregated by using the disulfide bonds, and the complex structure could affect the denaturation

and gel running (Reviewed in Stryer, 2001). The fusion proteins of human TTV and RoTTV showed slightly bigger in size than those of the predicted, and they could be affected by these factors.

In conclusion, we have described RoTTV1 RNA localisation in virus infected wood mice and predicted the virus replication site. This information is important for setting up the cell culture system and studying pathogenesis of RoTTV. Furthermore, we have identified the transcriptional profile and some RoTTV1 proteins were expressed in BHK-21 cells. These results can be applied for the study of the molecular biological characterisation to define the virus protein function and pathogenesis.

CHAPTER V. TTV REPLICATION IN VITRO

As RoTTV1 RNA was located in bone marrow, we concluded that this is a site of active viral replication. Wood mouse bone marrow derived macrophage and bone marrow stem cell cultures were grown for rodent TTV infection and transfection. Furthermore, concatemerised RoTTV complete genomes were used for transfection based on the reported porcine TTV infection study. The genomes were also transfected into BHK-21 cells.

5.1. Background to the study

5.2. Reagents for *in vitro* infection/transfection

5.3. Bone marrow derived macrophage/Wood mouse bone marrow derived stem cell

5.4. Type I IFN KO mouse embryo fibroblast (MEF)

5.5. Baby Hamster Kidney cell (BHK-21)

5.6. Comparative study of old and young passage of BHK-21 cells

5.7. Virus propagation

5.8. Discussion

5.1. Background to the study

One of the difficulties in carrying out research on TTV is the lack of a suitable cell culture system. Various types of the primary cells and cell lines have been used for human and porcine TTV studies and *in vitro* transcription has been shown in a variety of cell lines (Okamoto *et al.*, 2000; Kamahora *et al.*, 2000; Kamada *et al.*, 2004; Qiu *et al.*, 2005; Kakkola *et al.*, 2007; Muller *et al.*, 2008; Kakkola *et al.*, 2009; Huang *et al.*, 2012). However, virus production was not observed for porcine TTVs (Huang *et al.*, 2012).

In the human TTVs study, there have been the multiple attempts to propagate TTV in cell culture. TTV genome transfected cell cultures did not produce virus genomes into the culture supernatant (Kakkola *et al.*, 2007; Kakkola *et al.*, 2009). TTV genotype 1 DNA positive serum was added to Raji cell line and phytohemagglutinin (PHA) stimulated PBMC cell cultures and the copy number of TTV genomes in the culture supernatant was increased (Desai *et al.*, 2005; Mariscal *et al.*, 2002, Maggi *et al.*, 2001). However, the TTV infection in these cells was not sufficient to fully isolate virus. Firstly, in the cell culture system, the level of TTV replication was very low and the copy number of TTV DNA produced decreased over the studied time period. Secondly, the produced virus did not propagate to the intact cells efficiently.

Recently, human TTV replication was observed in 293TT cells, however these replicated viruses were smaller than original virus (de Villiers *et al.*, 2011). The smaller virus DNA was transfected into 293TT cells and DNA was detected in the cells and medium. However, the problem with this cell culture is that the copy number of TTV in the medium was low and by 10 days was decreasing. In addition, the transfected 293TT cells stopped DNA production of TTV when the cells were passaged (de Villiers *et al.*, 2011). Furthermore, infectious material was not observed in the culture supernatants and actual propagation has not been shown (de Villiers *et al.*, 2011).

Concatemerised TTSuV DNA failed to infect 12 different cell lines including porcine cell lines. However, concatemerised TTSuV DNA was directly inoculated to the piglet and the virus was

replicated (Huang *et al.*, 2012) indicating that concatemerised TTV can replicate in the cells.

In this chapter, we demonstrate the continuous *in vitro* replication of RoTTV and virus propagation. Based on the result of chapter IV, bone marrow derived cells were studied firstly and the other cell lines and the various infectious agents including concatemerised DNA were used to study RoTTV replication.

5.2. Reagents for *in vitro* infection/transfection

Several infectious reagents were prepared and constructed for the infection and transfection.

5.2.1. Liver homogenate

RoTTV infected liver was disrupted in PBS using a microtube pestle (Sigma-Aldrich). These samples were then homogenised using a fine Hypodermic needle (Approx. 15 gauge first and 23 gauge later to make fine homogenate) (Terumo) and hypodermic syringe (Terumo) and then centrifuged at room temperature for 1 minute for 5000 x g. Supernatant of liver samples was taken and filtered by sterilised 0.2µm filter Cartridge (Sartorius).

5.2.2. Faecal supernatant

Infectious reagent from faecal supernatant was prepared using the same method for DNA extraction from faecal supernatant (section 2.2.1.). This time, the centrifuged faecal supernatant was filtered by sterilised 0.2µm filter Cartridge (Sartorius).

5.2.3. Single copy of RoTTV complete circular genome.

Unique restriction sites were found in the RoTTV1 and RoTTV2 complete genome sequences (Spe I for RoTTV1 and HindIII for RoTTV2) and the inverted primers were constructed to contain these restriction sites (Table 2.15.4.1.). The amplicons were cloned (section 2.9.), plasmid preparations were performed and the plasmids were digested using the appropriate restriction enzyme (SpeI for RoTTV1 and HindIII for RoTTV2), and RoTTV complete genomes were purified by size fractionation on agarose gel (section 2.7.2.). Finally TTV complete genomes

were self-ligated by NEB T4 ligase (section 2.9.5.). The plasmids were transfected into the cells. A schematic representation of the construction of single copy of RoTTV complete circular genome is shown in Fig 5.2.3.1.

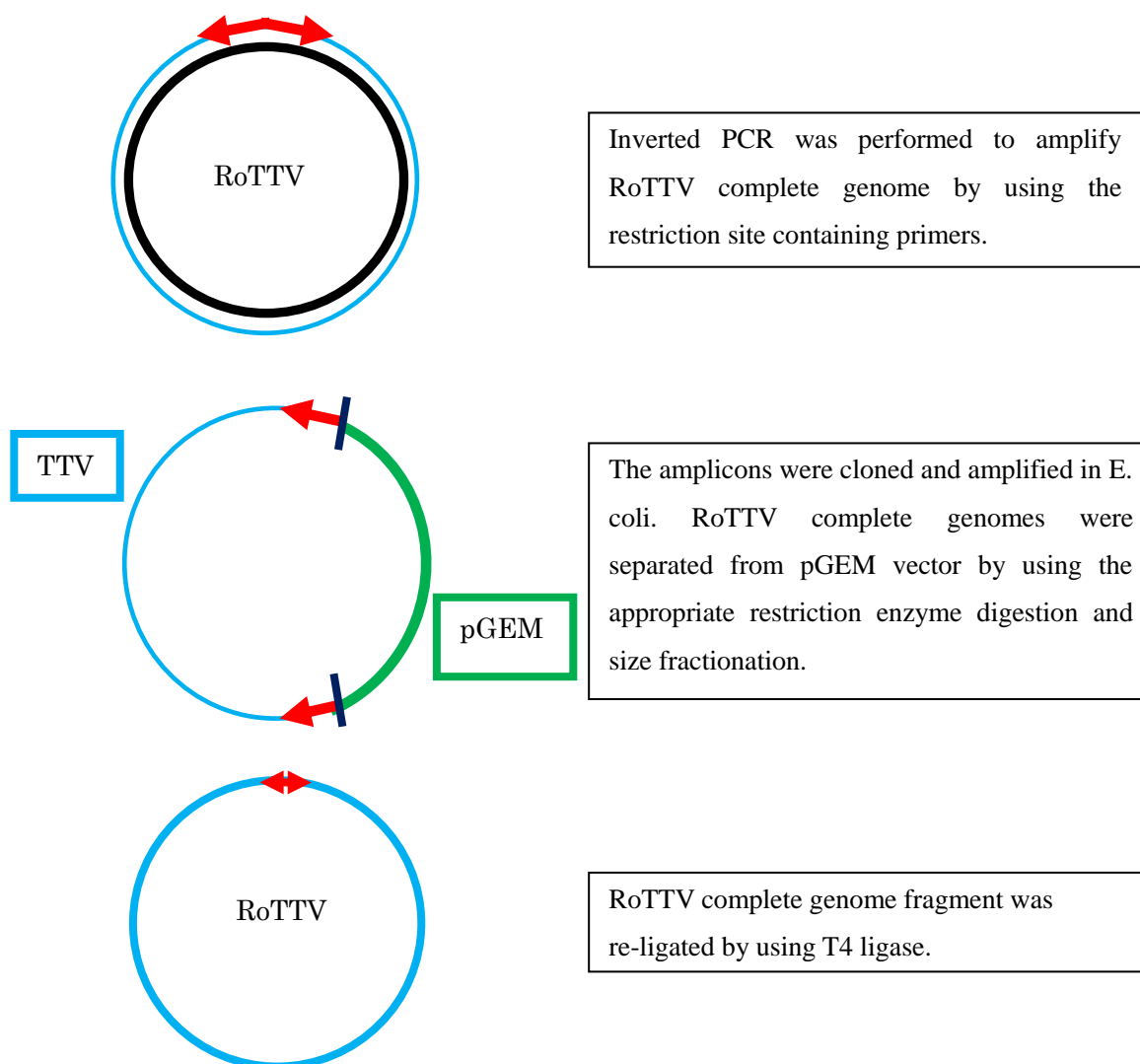


Figure 5.2.3.1. Construction of single copy of RoTTV genome

5.2.4. Construction of GFP/ RoTTV1

GFP/RoTTV1 was used for the confirmation of virus promoter function. GFP/RoTTV1 were constructed by the restriction site containing GFP and RoTTV1 primers (Table 2.15.4.2.). RoTTV1 primers were designed at the 5' end of ORF2 and such GFP should not disrupt any coding sequences shown in chapter IV. GFP genome sequence was amplified with the restriction sites by PCR from pGFP plasmid and the PCR fragment was cloned (section 2.9.). The RoTTV1 genome was amplified with the restriction sites by PCR and the PCR fragment was cloned (section 2.9.). GFP clones were digested by the appropriate restriction enzyme (Sph I for pGEM T easy vector and HindIII (Promega)) and run on agarose gels to purify the GFP fragments (section 2.7.2.). RoTTV1 clones were digested to linearise the plasmid by using the restriction enzyme (Sph I from pGEM T easy vector and HindIII (Promega) with Alkaline Phosphatase for removing phosphate groups and the products were purified by MinElute® PCR purification kit (section 2.7.1.). The GFP fragments and the linearised RoTTV1 clones were ligated to each other on the Sph I and HindIII sites by using NEB ligase (section 2.9.5.) and the ratio of RoTTV1 genome and GFP volume was 1:5. The ligated plasmids were cloned (section 2.9) and plasmid preparations were digested by Bam HI (Promega) and the digested products were self-ligated by NEB T4 ligase (section 2.9.5.). A schematic representation of GFP/RoTTV1 construction is shown in Fig. 5.2.4.1.

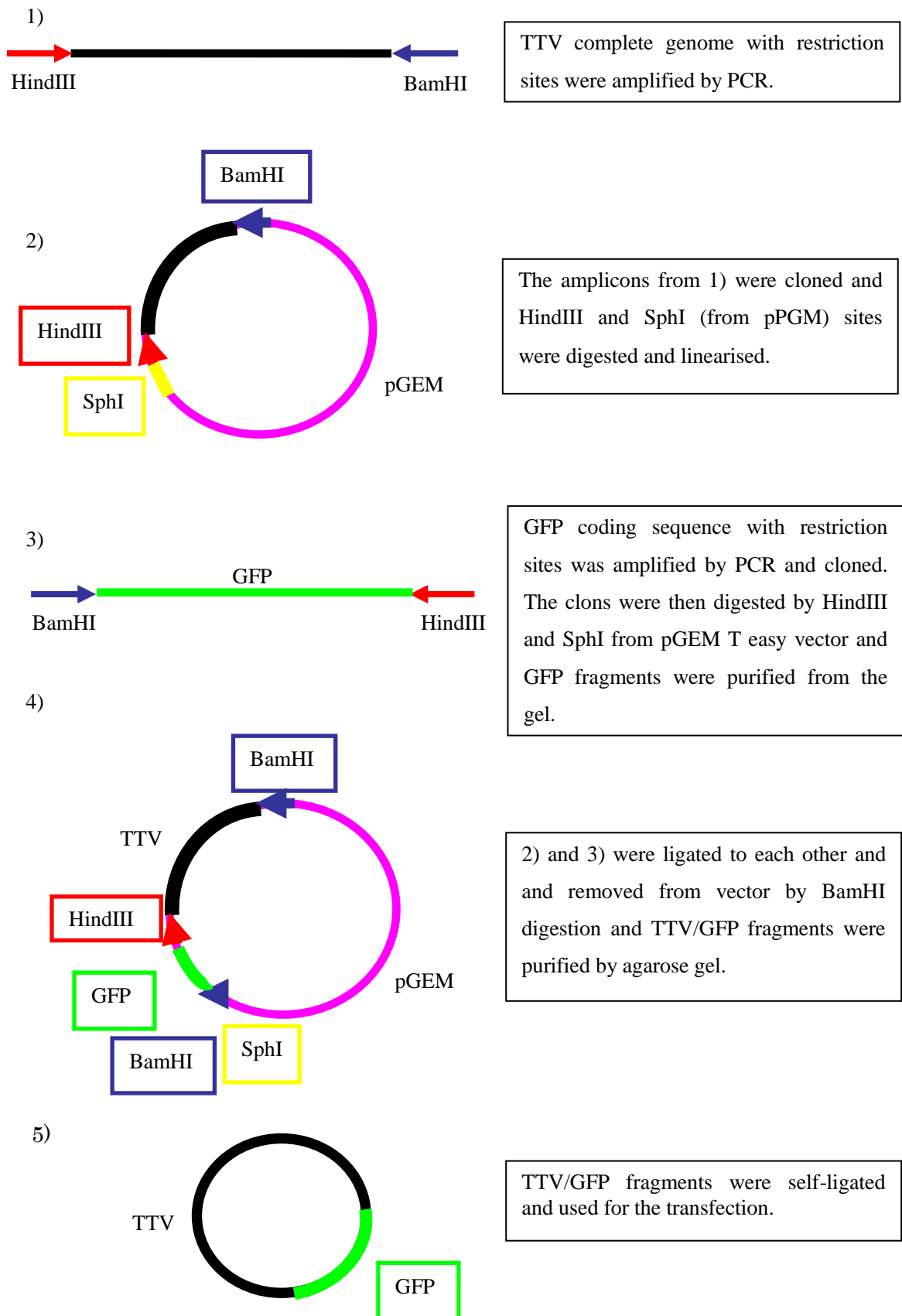


Figure 5.2.4.1. GFP/TTV construction.

By using concatemerised RoTTV complete genomes, the continuous complete RoTTV genomes were transfected into the cells and they were constructed by RCA (section 2.10.2.), restriction enzyme digestion (section 2.10.3.), and cloning (section 2.9.1.). The single copy of TTV complete genomes described in section 5.2.3. were used as the template of RCA. The amplified long TTV multiple copies of genome were digested by incomplete restriction enzyme digestion. Partially digested reactions were separated by agarose gel electrophoresis and gel slices containing more than two copies of complete genome were cut to extract concatemerised genomes (section 2.7.2.). The genomes were cloned into pGEM T easy vector (section 2.9.) (Fig. 5.2.5.1.).

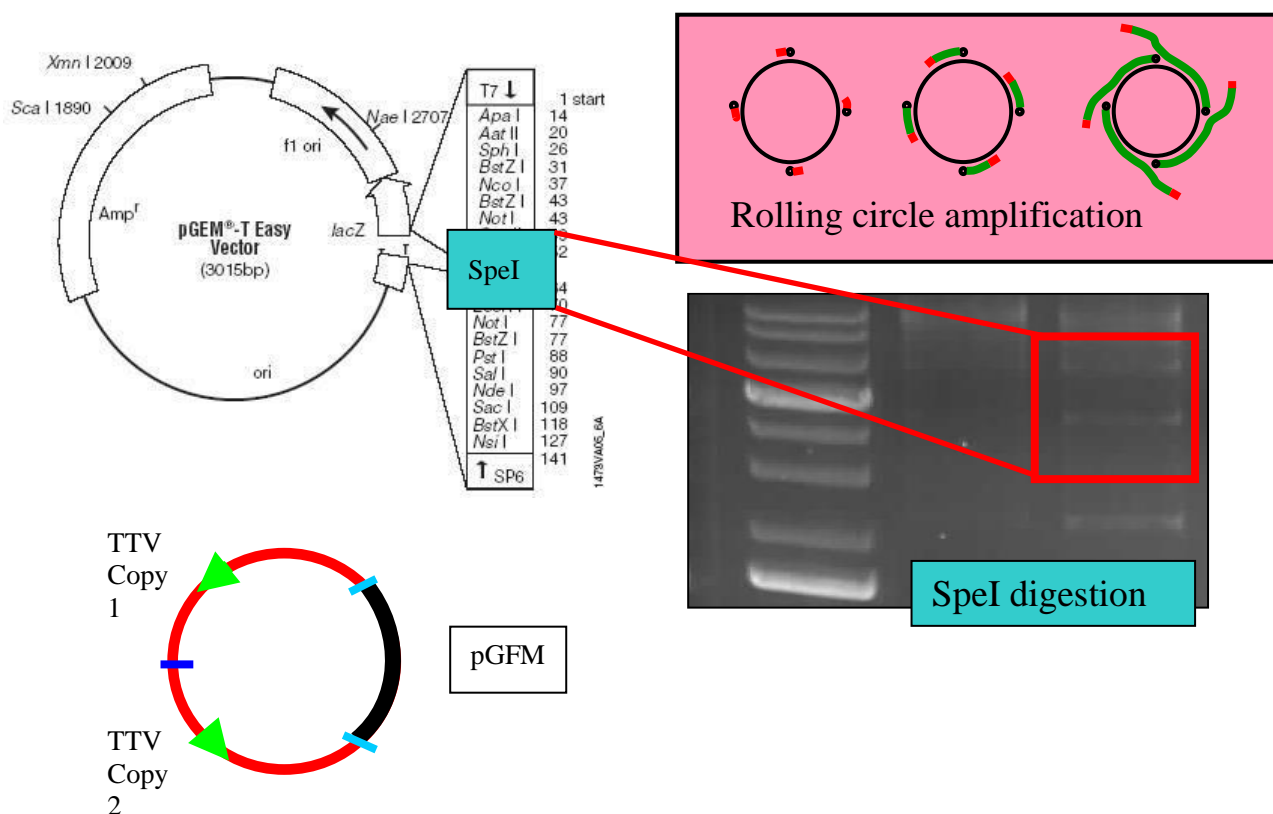


Figure 5.2.5.1. Concatemerised RoTTV genome clone construction. Circular RoTTV genomes were amplified by RCA. The amplicons were digested by incomplete restriction enzyme digestion. Two copies of complete genome were cut to extract concatemerised genomes and cloned.

5.2.6. Myc tagged concatemerised RoTTV

To reproduce myc tagged virus, myc tagged RoTTV1 complete genome was constructed by myc tagged primer (Table 2.15.4.3.) and RCA. The concatemerised genome was made by a similar method to 5.2.5. Single copy of myc RoTTV1 was prepared by the method discussed in section 2.15.5. and RCA (section 2.10.2.). The amplicons were incompletely digested, and the double size of RoTTV DNA was cloned (section 2.9.).

5.3. Bone marrow derived macrophage/bone marrow derived stem cell

Bone marrow derived macrophage

As shown by the results in chapter IV, RoTTV is predicted to replicate in the bone marrow and bone marrow derived macrophages were set up following the protocol described in section 2.15.2.1. Two types of bone marrow derived cell cultures were prepared; Type I interferon receptor KO mice and RoTTV negative wood mice. Bone marrow derived macrophages from both mouse species stimulated with LPS (E.coli $\Phi 55$ B5) were also used for the infectious experiment. In addition, two infectious agents were used for TTV infection; faecal supernatant and single copy RoTTV complete genomes.

Faecal supernatant was prepared from RoTTV infected wild (section 5.2.2.) mice. Serial dilutions of the samples were made and virus was detectable down to the 100 times dilution by PCR. Two, five, ten, and fifty times diluted faecal supernatants were added to the wells which contain the bone marrow derived macrophages (wood and type I IFN KO mice) and incubated 4 days. Total DNA was extracted from the entire cultures (both cells and supernatant). The virus copy number was compared to those of input faecal supernatant. Serial dilution was performed on DNA from input faecal supernatant and cell culture (medium and cells), and PCR was carried out on two replicate samples from each dilution. After compensation for different input volumes, RoTTV genome was observed until the same dilution (100 times) in both original samples and cell culture samples. Accordingly, there was no difference between the virus titer in input faecal supernatant and the virus titer in total DNA in cell culture, and hence no evidence that RoTTV

replicated in bone marrow derived macrophages.

Single copies of RoTTV1 and RoTTV2 genomes were constructed as described in section 5.2.3. and transfected into the bone marrow derived macrophages by using lipofectamine 2000 (Invitrogen) following the protocol shown in section 2.13.2. The pEGFP-N1 plasmid was used to monitor the transfection efficiency, but the transfection efficiency was low (<10%) and TTV RNA was not observed by RT-PCR.

Bone marrow derived stem cells

Bone marrow derived macrophages are the differentiated cells from the stem cells in bone marrow and RoTTV may require differentiated cells. Accordingly, the bone marrow stem cell cultures from the wood mice were prepared for RoTTV replication. Similar results to the bone marrow derived macrophages were also observed in bone marrow derived stem cells. Bone marrow derived stem cell cultures were set up following the protocol described in section 2.15.2.2. and bone marrow derived stem cells are shown in Fig. 5.3.1. Stem cells are fragile and the cells would be damaged by lipofectamine 2000 transfection. Therefore, SAFEctinTM-STEM transfection reagent (Deliverics) was used for the stem cell transfection (section 2.15.3.).

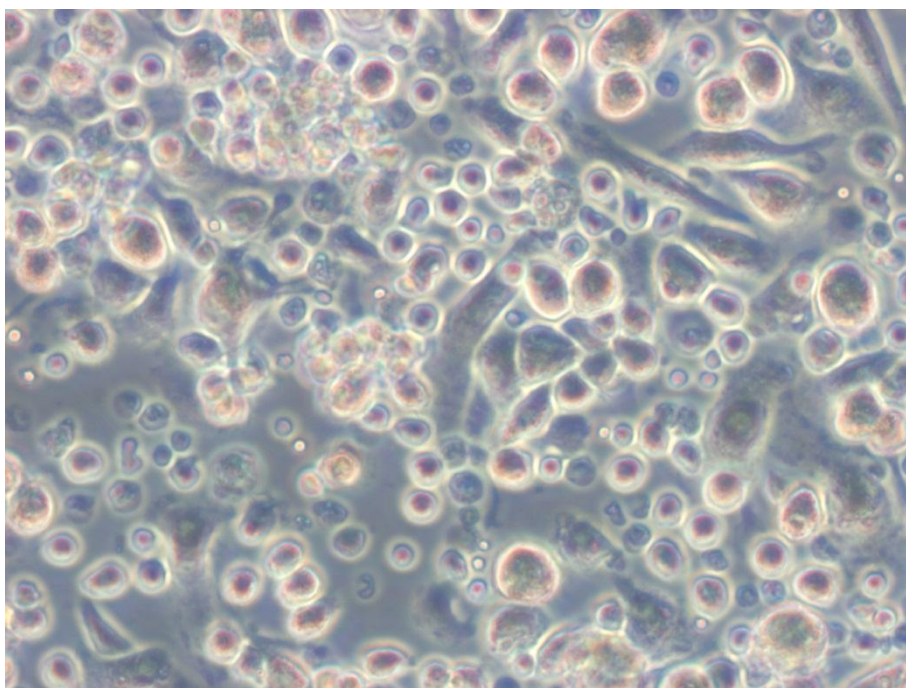


Figure 5.3.1. Bone marrow derived stem cell culture (Day 3). Stem cells were swelling at this time and used for the infection.

The single copy clone preparations of RoTTV were constructed as described in section 5.2.3. and transfected into the stem cells. Also, GFP/TTV plasmid was constructed as described in section 5.2.4. to confirm the virus promoter function and the transfection of plasmid into the stem cells. At the same time, pEGFP-N1 plasmid was used to monitor the transfection efficiency, but the transfection efficiency was low (<10%) and the promoter of GFP/TTV was not observed to function by fluorescence microscopy. RoTTV RNA was not observed in RT-PCR.

5.4. Type I IFN KO mouse embryo fibroblast (MEF)

Primary cells such as bone marrow derived macrophages and stem cells are very fragile and the transfection efficiency is low. A rodent cell which is less easily killed and shows good transfection efficiency was required to deliver the plasmid into cells and Type I IFN receptor KO MEF cells were therefore used for the infection and transfection studies. MEF cells were first confirmed as RoTTV negative by PCR (data not shown). Two infectious reagents were used for Type I KO MEF; the liver homogenate of RoTTV1 infected mice (section 5.2.1.) and the concatemerised RoTTV1 and RoTTV2 complete genome (section 5.2.5.).

Liver homogenate

In 6 well plate, 1.2×10^6 cells were infected with 0, 20 and 200 μ l of liver homogenate, MEF cells showed morphological change over a period of 2-3 days. These cells detached from the bottom of the tissue culture flask and culture medium colour changed from pink to red.

Serial dilution was performed on DNA from original homogenate and cell culture (medium and cells), and PCR was carried out on two replicate samples from each dilution. After compensation for different input volumes TTV genome was observed until the same dilution (100 times) in both original samples and cell culture samples. As virus was detected at the same dilution between original samples and cell culture samples, we believe that rodent TTV did not replicate in the cellular fraction of the culture. In a separate experiment, DNA was extracted from MEF cells only. No virus was observed in these cells indicating that TTV was not able to enter on attach to

MEF cells.

The concatamered RoTTV genome

The concatemerised DNA was constructed are described in section 5.2.5. The concatemerised RoTTV1 and RoTTV2 DNA plasmids were transfected into Type I IFN KO MEF in 24 well plates and the cells were incubated for 48 hours. Transfection efficiency was approximately 50-60% in pEGFP-N1 plasmid transfected MEF cells. Total RNA was extracted from MEF cells and mRNAs of RoTTV -1 and RoTTV2 were detected by RT-PCR by using RoTTV1 and RoTTV2 species specific primers (Fig. 5.4.1.). CPE was not observed in MEF cells.

In the previous study, the transcripts were detected in human and porcine cell lines (Okamoto *et al.*, 2000; Kamahora *et al.*, 2000; Kamada *et al.*, 2004; Qiu *et al.*, 2005; Kakkola *et al.*, 2007; Muller *et al.*, 2008; Kakkola *et al.*, 2009; Huang *et al.*, 2012) but evidence of productive TTV infection was not observed in these reports. To confirm virus replication, the medium of RoTTV transfected cells was taken for measurement of the virus genome copy number. Before measuring virus DNA, the transfected cells were passaged 5 times in 75 cm flask (BD Bioscience, UK) to remove as much as possible, the plasmids used for transfection (Fig. 5.4.2.). From the passage 6, 400µl of media was taken in day 0, day5 and day10 and the DNA was extracted. The virus replication was monitored by qPCR and results are shown in Fig. 5.4.3.

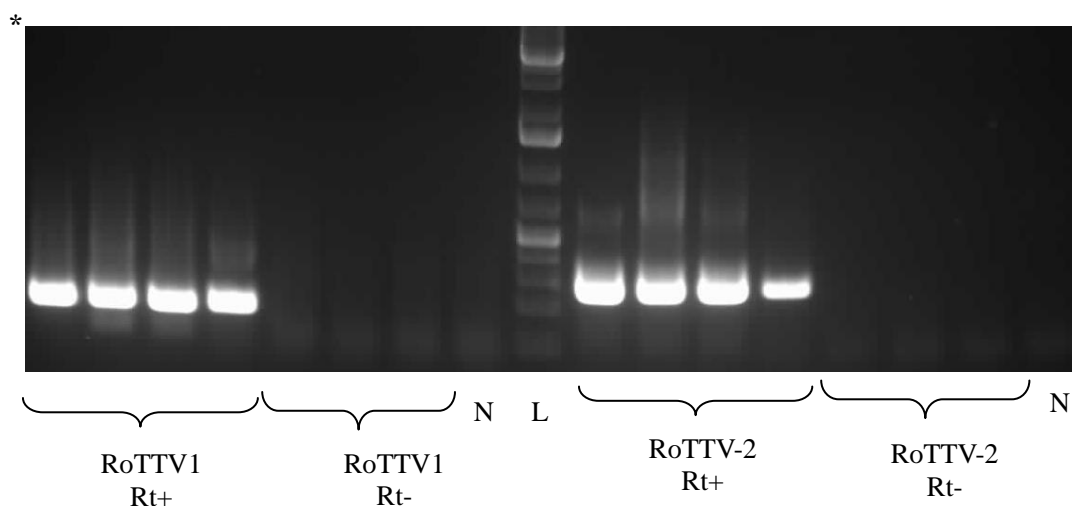


Figure 5.4.1. RoTTV1 and RoTTV2 transcripts were observed at 48 hours post-transfection by RT-PCR using RoTTV1 and RoTTV2 species specific primer. RT-PCR was performed in quadruplicates using same template cDNA. RoTTV1 PCR products; 180bp and RoTTV2 PCR products; 250bp. RT+; Reverse transcriptase including RT-PCR. RT-; Reverse transcriptase excluding RT-PCR. N; negative control. L; DNA ladder. *At 48 hours,

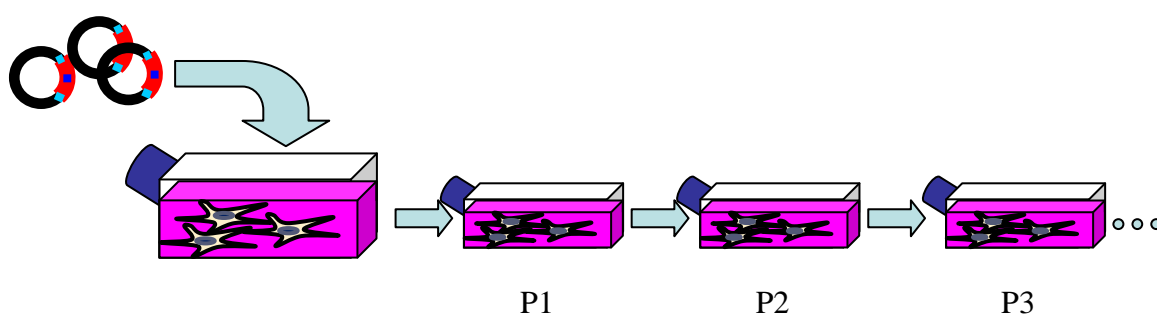


Figure 5.4.2. Experimental protocol of RoTTV1 virus replication study.

The transfected cells were passaged 5 times and 400μl of culture supernatant was taken in day 0, day 5, and day 10.

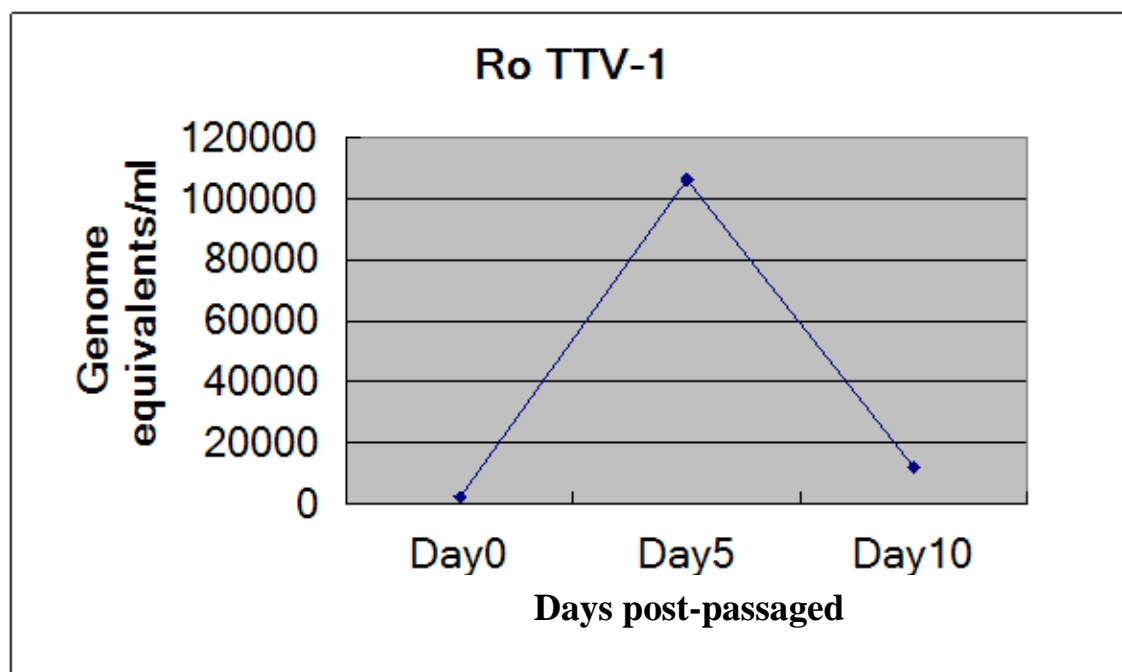


Figure 5.4.3. RoTTV1 viral DNA copy number in the supernatant culture fluid of the P5 transfected Type I IFN KO MEF. The virus titer was increased at day 5 and decreased by day 10.

The RoTTV1 viral load was high at day 5 post transfection, peaking at 1×10^5 genomes equivalents/ml and it then decreased to 1×10^4 genomes equivalents/ml. This indicated that while Type I IFN KO MEF do produce virus, they are not suitable for the constant virus replication.

5.5. Baby Hamster Kidney cell (BHK-21)

Type I IFN KO MEF did not show constant virus replication. MEF are a *Mus musculus* cell line, and it was concluded that *Mus musculus* is not a natural host of RoTTV based on the screening study of wild rodent species. However, it was also found that RoTTV was present in a number of rodent species other than *Mus musculus* (chapter III). Accordingly, another rodent cell line, Baby Hamster Kidney cells (BHK-21) was used for RoTTV infection. BHK-21 cells were first confirmed as RoTTV negative by PCR (data not shown).

RoTTV1 and RoTTV2 concatemerised genome plasmids were transfected into BHK-21 cells and the transcripts of RoTTV1 and RoTTV2 were also detected by RT-PCR (Fig. 5.5.1.). Transfection efficiency was approximately 80-90% in pEGFP-N1 transfected BHK-21 cells. The transcripts of

RoTTV2 were used as templates for RACE (section 4.3). Also, the transfected cells were passaged 5 times to remove the input plasmid and from passage 6, 400µl of the supernatant was taken on day 0, day 5, day 7, and day 13. Total DNA in 400µl was extracted from the supernatant and qPCR was used to monitor RoTTV replication. The result is shown in Fig. 5.5.2. RoTTV1 viral titer increased constantly up to 2.5×10^6 genome equivalents/ml by Day 13. BHK-21 cells were becoming over confluent on day 13. The media was used for the virus propagation experiment (section 5.7). No CPE was observed in RoTTV1 infected BHK-21 cells. These results indicate that BHK-21 showed the constant and high copy number of RoTTV1 replication. In cells transfected with concatemerised RoTTV2, viral DNA was not found to increase in the supernatant.

A c-myc tag was added to the end of ORF2-3 of RoTTV1 and myc RoTTV1 concatemerised DNA was constructed as described in section 5.2.6. The concatemerised DNA was transfected in BHK-21 cells and the cells incubated and the transcripts were observed in samples collected 2 days post-transfection. In addition, the cells were fixed and stained by anti-myc antibody based on the protocol 2.13.4 but no myc protein was detected in BHK-21 cells.

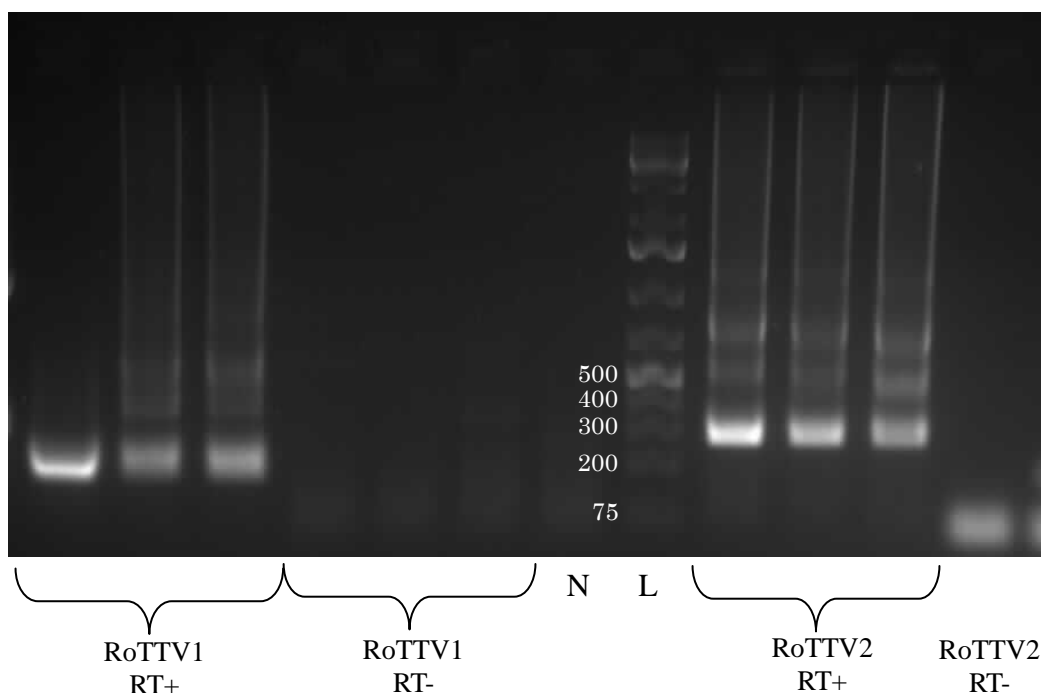


Figure 5.5.1. RoTTV1 and RoTTV2 transcripts were observed at 48 hours post-transfection by RT-PCR in the concatemerised DNA transfected BHK-21 cells. RT-PCR results using RoTTV1 and RoTTV2 species specific primers are shown and RT-PCR was performed in triplicate using the same cDNA. RoTTV1 PCR products; 180bp and RoTTV2 PCR products; 250bp. RT+; Reverse transcriptase including RT-PCR. RT-; Reverse transcriptase excluding RT-PCR. N; negative control. L; DNA ladder.

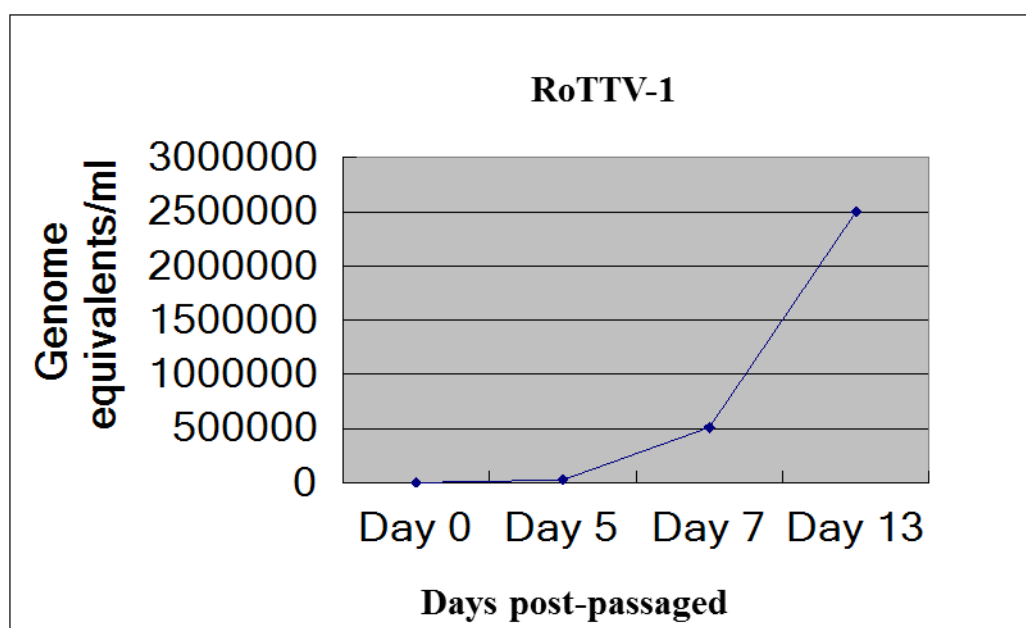


Figure 5.5.2. RoTTV1 viral DNA titer in the supernatant culture fluid of Passage 6 BHK-21 cells. The viral titer increased up to 2.5×10^6 genome equivalents/ml.

5.6. Comparative study of old and young passage of BHK-21 cells

Virus replication was confirmed by using the same BHK-21 cells used in section 5.5 (passage 30) (Old BHK21) and a lower passage batch of BHK-21 cells (Passage 10) (Young BHK). RoTTV1 concatemerised DNA was transfected into the two BHK-21 cell lines and the cells were passages 5 times with washing in PBS twice each passage to remove the input plasmids. The 400 μ l of supernatant was taken in day 0, day 1, day 3, day 4, day 5, and day 6, and virus replication was monitored by qPCR. The copy number of RoTTV1 in the medium of old and young BHK-21 cells is shown in Fig. 5.6.1.

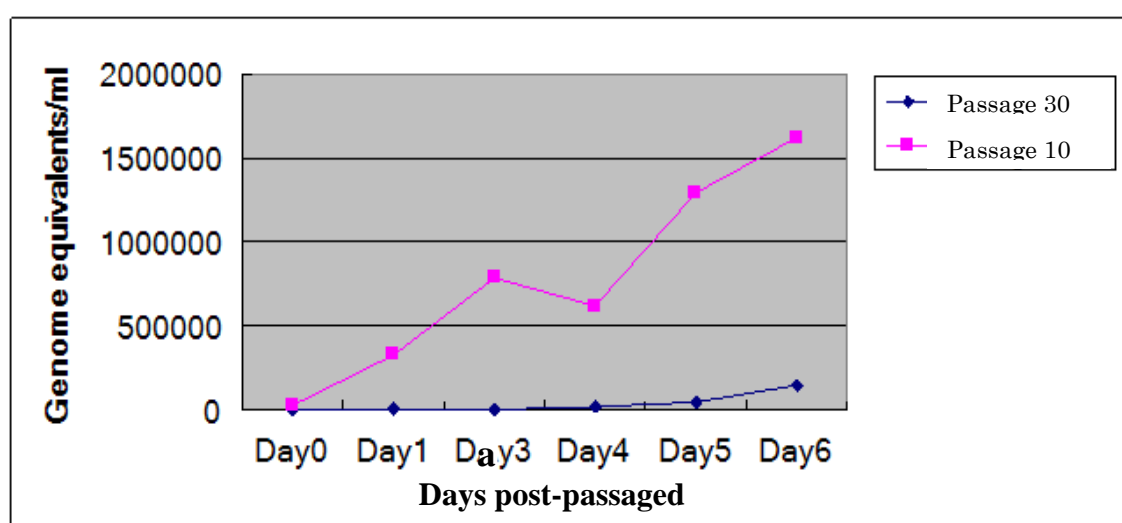


Figure 5.6.1. RoTTV1 viral DNA copy number in the culture supernatant of RoTTV1 transfected old and young passage of BHK-21. Young BHK-21 cells reproduced the high copy number of RoTTV1.

A huge difference of viral replication was shown between old BHK-21 cells and young BHK-21 cells. The RoTTV1 viral load of the supernatant culture fluid in old BHK-21 cells increased up to 1.5×10^5 genome equivalents/ml in Day6 and it was extremely slow compared to that of young BHK-21 cells. Young BHK-21 cells increased rapidly up to 1.6×10^6 genome equivalents/ml at Day 6. The cell appearance showed no difference and the growing speed of cells appeared similar between old BHK-21 cells and young BHK-21 cells.

5.7. Virus propagation

The supernatants of RoTTV1 transfected BHK-21 cells in section 5.5. was used to study the ability of presumed virus present in these supernatants to infect fresh BHK-21 cells. The supernatant containing (2.5×10^6 genome equivalents/ml) was put onto the fresh BHK-21 cells in a 24 well plates and the cells were incubated and samples were taken at 5 days and 11 days post-infection. The medium was old and nutritionally-poor media after the experiment described in section 5.5, but still supported growth of the BHK-21 cells. The cells started to show sign of damage and clumped but became confluent and were collected from the bottom of 2 wells of a 24 well plate per sample. The experimental design is shown in Fig. 5.7.1.

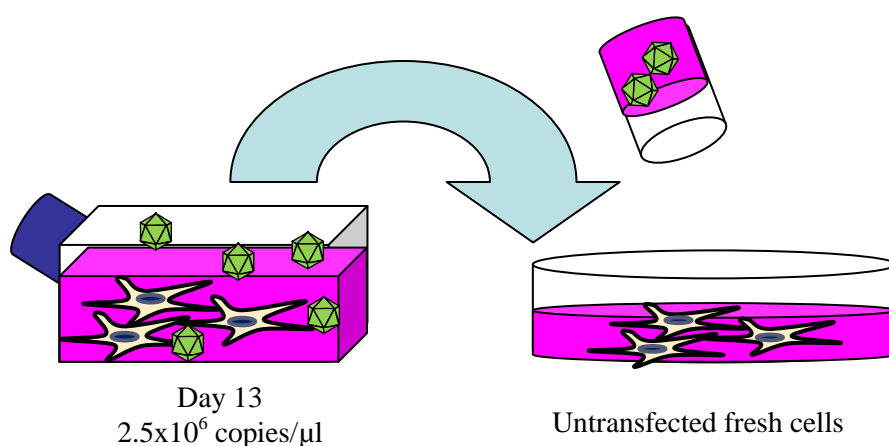


Figure 5.7.1. Experimental design of RoTTV1 propagation.

Supernatants positive for viral DNA were put onto the fresh BHK-21 cells and the cells were incubated for 5 days or 11 days.

Total RNA was extracted from BHK-21 cells at day 5 and day 11 post-infection and RT-PCR was performed in triplicate using RoTTV1 spliced area primer (Fig. 2.15.4.4.). These primers only detect the spliced transcripts and they can distinguish between the transcripts and virus DNA. RoTTV1 transcripts were not detected in day 5 BHK-21 cells and three non-specific bands were observed. However, strong RoTTV1 transcripts were detected in 2 of 3 replicates at day 11 (Fig. 5.7.2.).

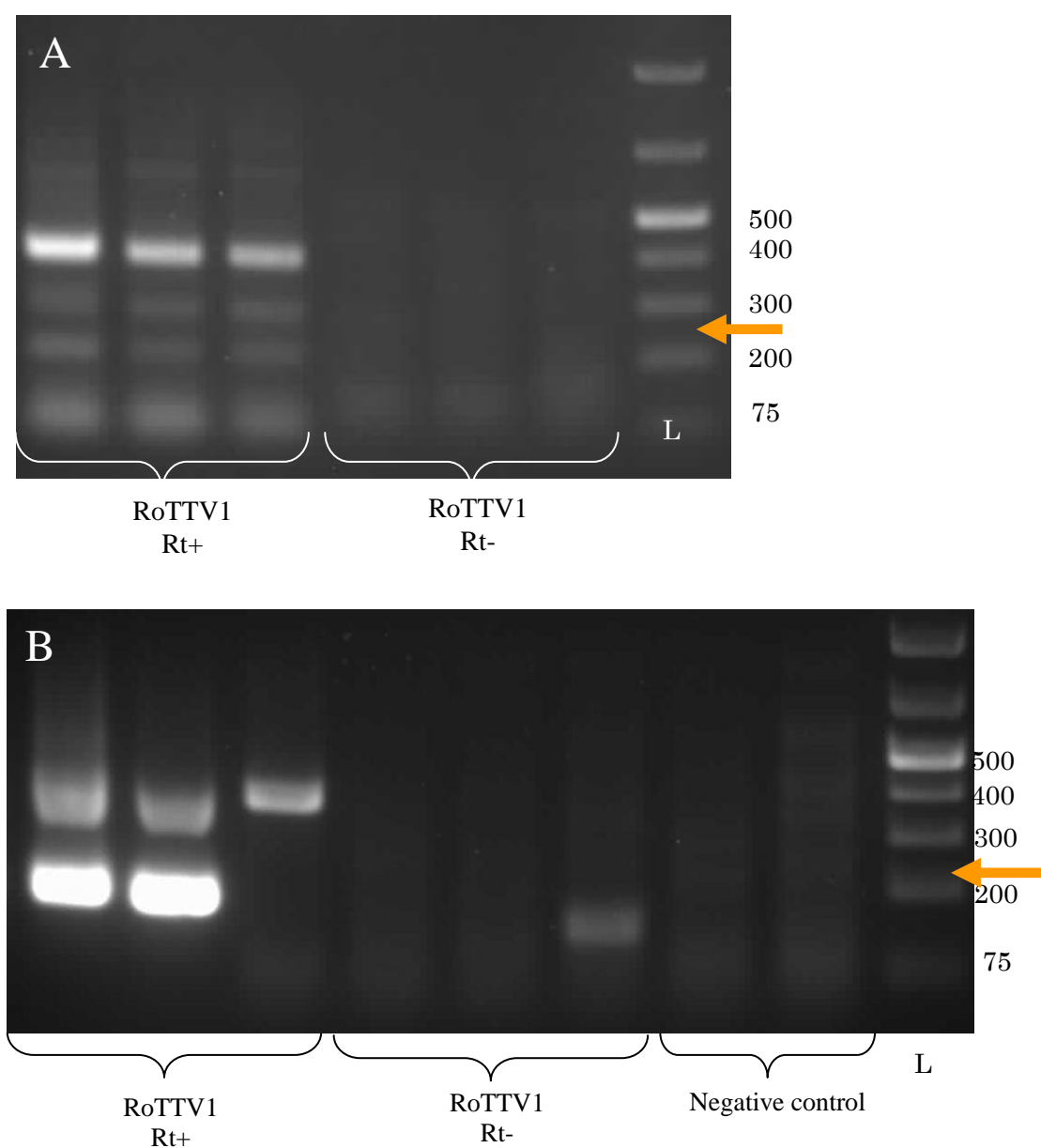


Figure 5.7.2. RT-PCR of RoTTV1 transcripts detecting the viral propagation. A; BHK-21 cell incubated for 5 days. B; BHK-21 incubated with viral DNA containing supernatants for 11 days. RT-PCR was performed in triplicate from a single RT reaction. RoTTV1 PCR products; 180bp. RT+; Reverse transcriptase including RT-PCR. RT-; Reverse transcriptase excluding RT-PCR. L; DNA ladder.

RoTTV1 transcript was not observed at day 5 (A) but it was observed in 2 of the 3 replicates taken at day 11 (B).

5.8. Discussion

In most areas of viral research, a cell culture system is highly beneficial for understanding the molecular biology of virus and establishing the model system to examine the functions of viral proteins and their interactions with the host cells. For human TTV cell culture, various cells were used for virus replication such as human peripheral blood mononuclear cells (PBMCs) (Maggi *et al.*, 2001; Mariscal *et al.*, 2002; Desai *et al.*, 2005), human hepatocyte cell lines (Desai *et al.*, 2005), leucocyte cell line (Desai *et al.*, 2005), and 293 TT cells (de Villiers *et al.*, 2011). However, the level of virus replication was very low and continuous propagation was not shown in these cells. Similar studies were also performed for porcine TTV and 12 cell lines including BHK-21 and porcine cell lines were used for attempts to replicate TTSuV2 infection (Huang *et al.*, 2012) but virus was not detected in the supernatant of culture fluid of TTSuV2 transfected cells. Therefore, cells which produce virus constantly are required for TTV research. For the study of virus, various rodent cell lines are available and this is one of the reasons why RoTTV represents an attractive model system.

Before the study, all primary cells and cell lines were screened by species specific RoTTV primer or pan-TTV primer. TTV is widely distributed in human and animal population, and the reagents for the cell culture and porcine cell line have shown contamination with TTV (Kekarainen *et al.*, 2009; Teixeira *et al.*, 2011), although some porcine cell lines have been confirmed as TTV free (Huang *et al.*, 2012). Therefore, wood mice used for the primary cell line, Type I IFN receptor KO MEF, and BHK-21 cells were screened before the experiment and we found they were free of RoTTV.

In this study, three cell types were used for RoTTV infection and these cells have some potential advantages and disadvantage for RoTTV replication. The primary cells such as bone marrow derived macrophage or stem cell were used for cell culture. In chapter III, we found that more than 90% of wood mice had RoTTV1 or RoTTV2 and wood mice were indicated as a natural host of RoTTV. Furthermore, in chapter IV, RoTTV RNA was detected in bone marrow and it

was concluded that bone marrow was main replication site of RoTTV. Based on these results, bone marrow derived macrophage and stem cell cultures were prepared for the RoTTV1 infection. RoTTV replication was not observed in these primary cell lines but it could be related to the agents used for RoTTV infection. Faecal supernatant was used for the infection but the viral copy number of faecal supernatant was low (virus was only detectable until 10^2 dilution by PCR) and also a longer incubation period might be required for RoTTV infection. In addition, wood mice stem cell culture had almost all factors for RoTTV infection such as natural host derived cell culture and target cells for infection, but the primary cells showed low transfection efficiency and the viral replication was not observed. However, it is possible that if the supernatant of culture fluid of RoTTV1 infected BHK-21 (section 5.7) was used for infection of these primary cells, and the virus might replicate in these cells.

The next candidate was Type I IFN receptor KO MEF cells. After primary cell culture experiment, the cell lines which have shown good transfection efficiency were chosen for RoTTV replication. Furthermore, this cell line lacks the Type I IFN receptor and is more likely to support virus replication. Before the transfection experiments, the liver homogenate of RoTTV infected wild wood mouse was used for RoTTV infection. MEF cells incubated with the liver homogenate were damaged and detached from the bottom of the tissue culture flask. However, RoTTV virus replication was not observed in the cell culture and it was hypothesised that the liver homogenate contains strong liver enzymes which damaged the MEF cells.

For the transfection of Type I IFN receptor KO MEF cells, the concatemerised RoTTV genome was constructed for the infection. The advantage of the concatemerisation is the vector does not disturb RoTTV genome and the continuous complete genome sequence was transfected into the cells (Fig. 2.15.7.1.). The concatemerised RoTTV1 and RoTTV2 produced viral transcripts in transfected MEF cells. Similarly, transcripts were detected in the various cell lines used in human and porcine TTV studies (Okamoto *et al.*, 2000; Kamahora *et al.*, 2000; Kamada *et al.*, 2004; Qiu *et al.*, 2005; Kakkola *et al.*, 2007; Muller *et al.*, 2008; Kakkola *et al.*, 2009; Huang *et al.*, 2012).

This indicated that the promoter of RoTTV worked correctly and was making the transcripts for protein synthesis. However, the secreted RoTTV1 in the supernatant of culture fluid of RoTTV1 MEF cell decreased by day 10 and RoTTV2 did not show any replication of virus in supernatant. In chapter III, RoTTV infection was not observed in *Mus musculus* in wild rodents which may indicate that the cells of *Mus musculus* may lack the function to reproduce RoTTV continuously.

BHK-21 cells were the most successful cell line for RoTTV replication in this study and have several advantages. Firstly the transfection efficiency of BHK-21 cell was high (more than 80-90%) and almost all cells gained the concatemerised RoTTV1 genomes. Secondly, BHK-21 cells are not *Mus musculus* cell lines. Thirdly, BHK-21 cells are known to be widely permissive to the replication of several viral species (Martin *et al.*, 2010; Huang *et al.*, 2011; Park *et al.*, 2013). RoTTV1 concatemerised DNA transfected BHK-21 cells still reproduced the high copy number of RoTTV1 even after 5 passages and the replication was continued at the end of the experiment. Human TTV replication by using human cell line was observed but the copy number was low (Chang liver cell; 2.2×10^2 genome equivalents/ml) (Desai *et al.*, 2005). Furthermore, another problem of TTV cell culture study is the difficulty in achieving continuous replication. It was reported that PHA stimulated PBMCs showed 3.6×10^4 genome equivalents/ml once but the virus titer dropped down immediately (Desai *et al.*, 2005). In this study, RoTTV1 replicated continuously and it is indicated that BHK-21 cells is the best cell line to support RoTTV1 replication.

RoTTV1 replicated in Type I IFN receptor KO MEF and BHK-21 cell but RoTTV2 replication was not observed. This species difference was also observed in human and porcine TTV infection. The various genotypes of human TTV were transfected into Hodgkin's lymphoma and virus replication was found only in TTV-HD types (de Villiers *et al.*, 2011). Also, the concatemerised TTSuV1 and TTSuV2 genomes were inoculated into piglets and only TTSuV2 showed replication. It is suggested that the promoter/enhance activity of the different TTV genotypes was variable depending on the types of cells and the different genotypes may have

different transcriptional regulation activity in TTVs (Kamada *et al.*, 2004). Therefore, the different species of RoTTV may show different host cell tropism.

Also, RoTTV1 may infect the new host more easily than RoTTV2. In chapter III, RoTTV1 showed higher genetic diversity than RoTTV2 and RoTTV1 complete genomes were isolated from wood mice, field voles and bank voles.

RoTTV1 replicating BHK-21 cells did not show CPE. CPE related to TTV infection was reported in Chang liver cells and the focal accumulations of dense and rounded cells were observed (Desai *et al.*, 2005). TTV is widely distributed in human and animal population and it is believed that TTV does not have any strong pathogenicity. Also, to establish persistent infection, RoTTV cannot destroy every host cell. It is known that viruses apply two common mechanisms to achieve and maintain a persistent infection; (1) evasion of host immune response (Mathur *et al.*, 1986) and (2) change of virus replication and transcription style which reproduces incompetent or defective virus unable to lyse the host cells (Huang and Baltimore, 1970; Brinton *et al.*, 1982; Poidinger *et al.*, 1991; Meyer and Southern, 1997). The later mechanism was observed in the various viruses such as Sendai virus (Chandrika *et al.*, 1995), Sindbis virus (Weiss *et al.*, 1983), Semliki Forest virus (Atkinson *et al.*, 1986; Barrett & Dimmock, 1984), and vesicular stomatitis virus (VSV) and rabies virus (Cave *et al.*, 1985; Holland *et al.*, 1976) and a recent TTV study also suggested that TTV may use the similar mechanism. TTVs transfected 293TT cells reproduced the subviral molecule of TTV (μ TTV) (de Villiers *et al.*, 2011). Also, TTVs intragenomic rearrangement was suggested by the same group and plant viruses of the Geminiviridae showed the similar subviral genomes (Leppik *et al.*, 2007). TTV may establish the persistent infection by using these subviral molecules. TTVs are thought to infect 90% of healthy individuals and established the persistent infection in the hosts. TTVs may use the non-pathogenic method for the replication.

BHK-21 cells showed a huge difference of virus replication between young and old passaged

cells. TTV replicated rapidly in young passage and the copy number was higher than that of old passage of BHK-21 cells. Old passage cells are believed to divide slowly and eventually arrest cell division (Campisi and d'Adda di Fagagna, 2007). It is predicted that TTVs use the host cell replication mechanism and TTV would infect the highly replicating cells. Accordingly, when the host cells stop cell division, TTVs would also stop replication. It was confirmed that TTV replication was completely blocked by aphidicolin (an inhibitor of eukaryotic nuclear replication) treatment of 293 T cells. There are two hypotheses for the end of cell line division; anti-tumour and anti-cancer suppressive mechanisms function or loss of regenerative capacity (Campisi and d'Adda di Fagagna, 2007). These cellular changes such as the aging or alternation of gene expression would hamper the virus replication and young BHK-21 cell actively replicated and supported the virus replication. However, the replication speed of old and young BHK21 cells looked to be similar in this study. BHK-21 was originally developed from polyoma virus transformed hamster kidney cells in 1962 (Macpherson and Stoker. 1962) and is highly susceptible for the various virus species (Stoker and Macpherson. 1964). Furthermore, BHK-21 cells showed the high growth rate and are used in various studies including herpesvirus study in our lab (Geere *et al.*, 2006) indicating that the stocks available are likely to be highly passaged cells. Accordingly, it is difficult to identify BHK-21 cells quality in this study. Old BHK-21 cells would be exposed to trypsin and other environmental factors more often than young BHK-21 cells, but it was considered that these stimulations and agings in tens of passages would not show a huge difference of virus replication. Further study is required to understand RoTTV-1 replication *in vitro*.

Some viruses such as Simian Human Immuno Deficiency virus (SHIV) and West Nile Virus (WNV) increased the virus replication after about 20 passages and it was indicated that these viruses took time to adapt the cell culture (Ciota *et al.*, 2012; Kwofie and Miura. 2013). On the contrary, RoTTV1 increased constantly and it was indicated that RoTTV1 does not need further adaptation for replication in BHK-21 cells.

In this study, the supernatants of RoTTV1 transfected BHK-21 cells were infectious and RoTTV1 in the supernatant propagated infection to the fresh BHK-21 cells resulting in detectable loads of viral RNA being produced. The primers for the RT-PCR were designed to cover the splicing site of RoTTV1 and only amplified RoTTV1 cDNA would create the correct size of band (Fig.5.7.2.). For TTV study, TTVs propagation is a major difficulty and human TTVs and porcine TTVs have not been propagated into intact cells. Accordingly, it was first success to demonstrate TTV propagation *in vitro* study.

Recently, a study investigating TTV replication and propagation was published and the paper showed the subviral molecules in TTV genome transfected 293TT cells (de Villiers *et al.*, 2011). However, we question two claims of this study; the constant viral replication and viral propagation. In the study, the cells transfected with TTV genomes reproduced subviral molecules of TTV (μ TTV) but the number produced decreased soon after the transfection. This indicated that 293TT cells temporarily reproduced the virus but could not sustain continuous replication. Also, the subviral molecules were transfected into 293TT cells to produce other subviral molecules, and this was defined as viral propagation in the paper. However, if it is propagation, the viruses in the supernatant or frozen transfected cells would have to infect fresh cells as we have. We believed that in the present study, we have shown that RoTTV1 actually replicated in BHK-21 cells and have shown true virus propagation by using the virus containing supernatant of culture fluid to infect fresh cells. For the future study, RoTTV should be analysed by using electron microscopy (EM) to confirm the virus replication.

In conclusion, RoTTV1 replicated in BHK-21 cells and the replicated virus can be propagated into fresh uninfected cells. This culture supernatant will be used in *in vivo* studies (chapter VI). Also, these experiments demonstrate a suitable cell culture system providing tools for exploring the virus-cell interaction and molecular and biochemical mechanism of TTV infection.

CHAPTER VI. IN VIVO EXPERIMENTS OF TTV INFECTION IN WOOD MICE AND TYPE I IFN RECEPTOR KO MICE

Only a few papers have reported *in vivo* infection studies. One such study reported that concatemerised TTV complete genomes replicated in one month old piglets. The rodent TTV infectious experiment was planned based on the protocol of this paper. Following our demonstration that RoTTV1 can be isolated from BHK-21 cells, this virus was inoculated into wood mice.

6.1. Background to the study

6.2. TTV infection using concatemerised genome in wood mice/Type I IFN KO mice

6.3. TTV infection using cell culture supernatant in wood mouse

6.4. Discussion

6.1. Background to the study

As there is no established TTV cell culture system, very few studies on TTV infection *in vivo* have been reported. The majority of published studies used TTV infected samples such as the serum of human TTV1a infected patient (Tawara et al. 2000), TTSuV1 containing porcine serum (Krakowka and Ellis. 2008), and TTSuV2 containing liver homogenate (Mei *et al.*, 2011) as the infectious agent and the pathological findings from these studies may show the pathological character of other pathogens present in the inoculum. Therefore, the virus isolation in a cell culture system or by other methods is needed in order to study TTV infection *in vivo*.

Recently, an *in vivo* porcine TTV study was performed by using the concatemerised TTSuV2 complete genome (Huang *et al.*, 2012). In these experiments, the concatemerised TTSuV2 DNA was directly inoculated into the lymph node or muscle of 40 days old piglets and virus replication was observed and viral copy number is increased continuously (Huang *et al.*, 2012).

In this chapter, *in vivo* RoTTV1 infection experiments were performed by two methods; *in vivo* transfection of RoTTV1 concatemerised DNA and infection with cultured RoTTV1 virus in BHK-21 cell culture supernatant. As shown by the results in chapter IV, the lung was concluded as a site of RoTTV1 replication and these infectious reagents were inoculated intranasally.

6.2. TTV infection using concatemerised genome in wood mice/Type I IFN receptor KO mice

Based on the previous TTSuV2 study (Huang *et al.*, 2012), an infectious experiment was performed and we applied two points from this paper; 1) young animals were used for the infection, and 2) concatemerised DNA was inoculated. In addition, two points were added to this study; 3) as we have confirmed that the lung is a replication site of RoTTV1, the infectious agent was inoculated intranasally, and 4) to facilitate the *in vivo* transfection, the transfection agent, polyethylenimine (PEI) was used.

Initial results/*In vivo* transfection of wood mice colony

The wood mice were confirmed as RoTTV negative by using PCR before the study. Concatemerised RoTTV1 genome was constructed as described in section 5.2.5. and 1 µg of RoTTV1 concatemerised DNA and 0.1M PEI reagent were mixed in a 1.5 ml microcentrifuge tube (microtube) as described in section 2.16. The mixture was incubated for 15-20 minutes at room temperature and then inoculated intranasally into 4 female wood mice aged 23-70 days (Table 6.2.1.). One month post-infection, lung, liver, spleen, kidney, intestine, and bone marrow were removed and PCR was performed using DNA from lung, liver and spleen, and RoTTV1 species specific primers. The PCR results are shown in Fig. 6.2.1.

*No	TTV Species	†Sex	Infection Duration	Age of infection	DNA	Positive tissue
Mo1.1	RoTTV-1	F	28 days	70 days	Positive	lung, liver, intestine
Mo1.2	RoTTV-1	F	28 days	70 days	Positive	lung,liver,speen, kidney,intestine
Mo1.3	RoTTV-1	F	28 days	70 days	Positive	Lung
Mo1.4	RoTTV-1	F	28 days	23 days	Positive	Lung, live, spleen

Table.6.2.1. Initial experiment of RoTTV1. * Mice identification number. †M=male, F=female.

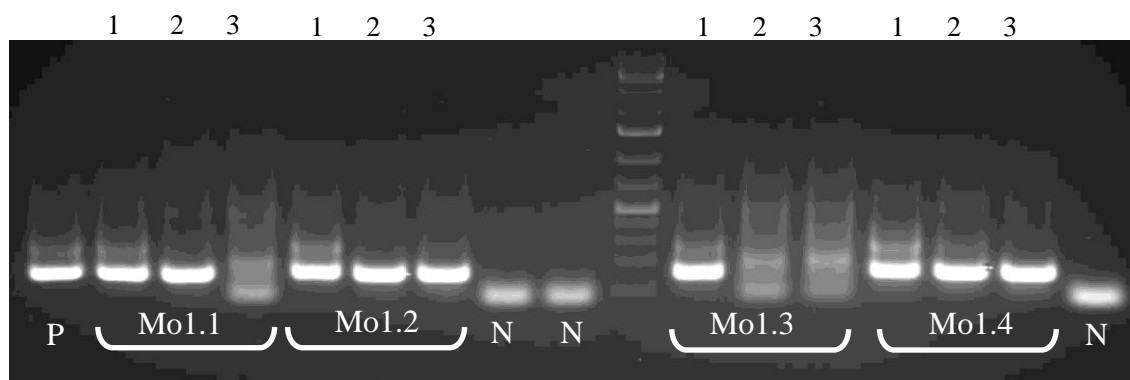


Figure 6.2.1. Screening of *in vivo* transfected wood mice by PCR using RoTTV1 species specific primer.

P: positive control, N: negative control, M: wood mouse

Lane1: lung, Lane2: liver, Lane3: spleen.

All transfected mice showed RoTTV1 DNA remaining in the lung and RoTTV1 DNA was also detected in the liver and/or spleen of mice 1, 2, and 4 one month post-infection.

RoTTV1 DNA was detectable in all 4 mice and TTV DNA was present in spleen or/and liver in 3 mice. DNA inoculated intraperitoneally was usually cleared from the mice within one month (data not shown) the result suggested that PEI functioned for *in vivo* transfection and RoTTV1 concatemerised DNA spread to the various tissues. However, RNA was not detectable in these tissues.

The virus copy number in the various tissues was measured by using qPCR and the results are shown in Fig. 6.2.2. In all animals, the lung showed the highest DNA copy number of RoTTV1, Mouse 1.4 had 1.74×10^6 copies/ μg in the lung and a high copy number was also observed 4×10^3 copies/ μg in liver DNA. However, this mouse showed a high virus copy number only in the lung and liver and the viral copy number was low in other tissues. Conversely, mouse 1.2 showed a relatively low copy number in lung compared to that of mouse 1.4, but viral DNA level was high in spleen, kidney, and intestine indicating that even in the same species and age, wood mice showed different viral DNA distribution in each tissue.

Due to the limitation of the mice, there were no mice used for the negative control and this was the pilot study.

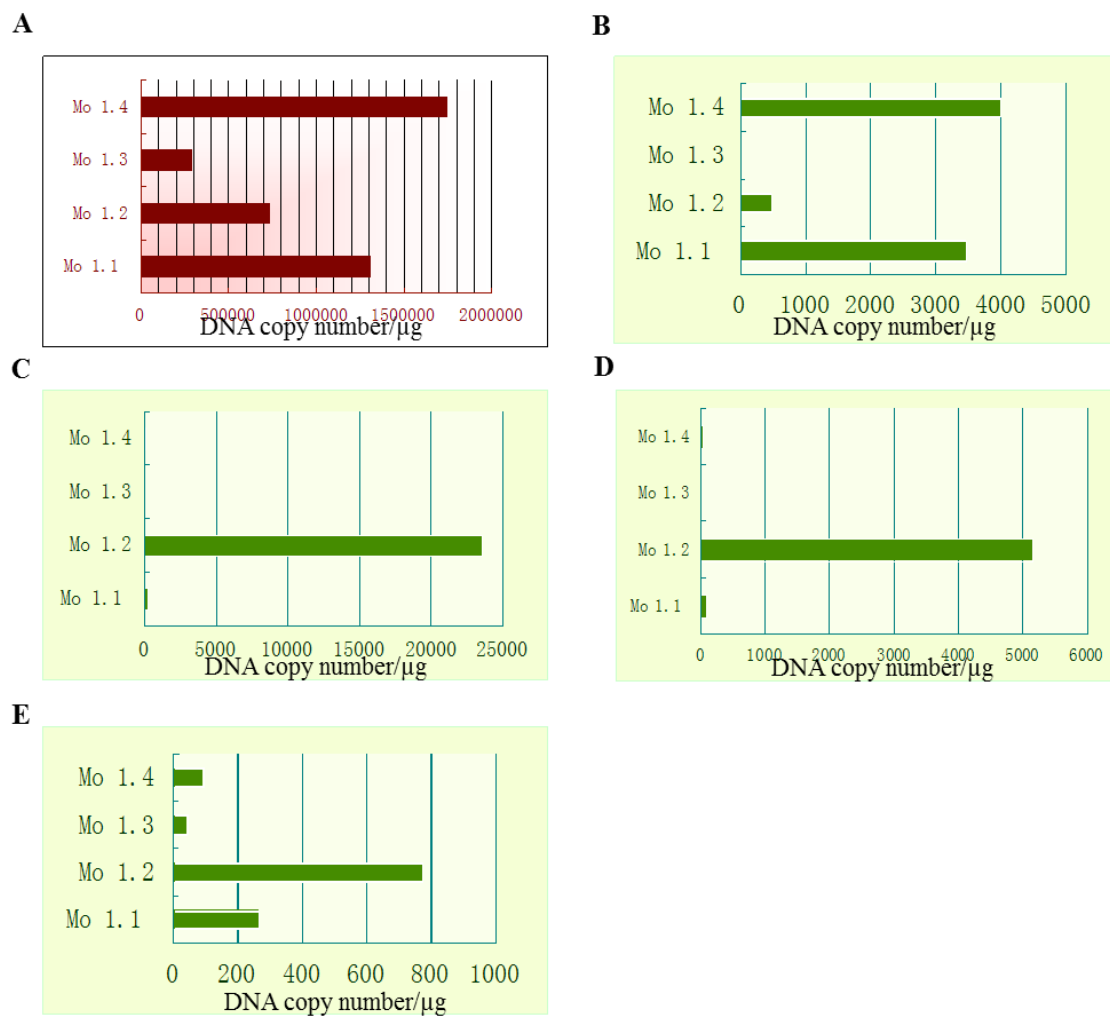


Figure 6.2.2. RoTTV viral DNA copy number and distribution in systemic organ.

RoTTV1 copy number per µg of input DNA was shown in each tissue. The high viral copy number was detected in lung and liver DNA of mouse 1.4 and RoTTV1 DNA was detectable in the various tissues of mouse 1.2. (A) Lung DNA, (B) Liver, DNA (C) spleen DNA, (D) kidney DNA, and (E) intestine DNA.

Second experiments/*in vivo* transfection of wood mice colony

A second experiment was also performed by using two groups with longer infection periods. Four females and four males aged 45-49 days (Table 6.2.2) were infected intranasally with 1µg of RoTTV1 concatemerised DNA. For each of the 2 time points (1 and 2 months post-infection), four mice were infected. The results of the second experiment are shown in Table 6.2.2. The lung, liver, spleen, kidney, intestine, and bone marrow were removed from four mice (Mo1.7-Mo1.10) and PCR was performed using DNA extracted from lung, liver, and spleen, and RoTTV1 species specific primers. One month post-infection, lungs (except for Mo 1.9) were negative for RoTTV DNA suggesting that the input concatemerised DNA was cleared. At two month post-infection, the lung from all four mice (Mo1.11-Mo1.14) showed RoTTV1 DNA. Although TTV DNA was detectable in lung of these mice, systemic viral DNA distribution and RoTTV1 RNA was not observed in these mice†

* No	TTV Species	†Sex	Infection Duration	Age of infection	DNA	Positive tissue
Mo1.7	RoTTV-1	M	33 days	45 days	Negative	
Mo1.8	RoTTV-1	M	33 days	45 days	Negative	
Mo1.9	RoTTV-1	M	33 days	46 days	Positive	Lung
Mo1.10	RoTTV-1	M	33 days	49 days	Negative	
Mo1.11	RoTTV-1	F	61 days	46 days	Positive	Lung
Mo1.12	RoTTV-1	F	61 days	46 days	Positive	Lung
Mo1.13	RoTTV-1	F	61 days	46 days	Positive	Lung
Mo1.14	RoTTV-1	F	61 days	46 days	Positive	Lung

Table 6.2.2. Second experiment of RoTTV1 *in vivo* transfection. * Mice identification number and there were two groups used for the infection; Mo1.7-1.10 for one month post-infection and Mo1.11-Mo1.14 for two post-month . †M=male, F=female.

***In vivo* transfection of Type I IFN KO mice**

An *in vivo* experiment was also performed using Type I IFN receptor KO mice. Although lab mice were *Mus musculus* (which is not natural host of RoTTV), these mice are Type I IFN receptor deficient and RoTTV1 may be more likely to infect them. The experimental protocol was the same as that used for wood mouse infection and 1µg of concatemerised RoTTV1 DNA and PEI complex was intranasally inoculated into 3 male Type I receptor KO mice aged 30 days. At one month post-infection, the lung, liver, spleen, kidney, intestine and bone marrow were removed from these lab mice and PCR was carried out using DNA extracted from lung, liver, and spleen, and RoTTV1 species specific primers. The PCR result is shown in Fig. 6.2.3. RoTTV1 DNA was detected in the lungs of all mice and in the spleen of mouse 1. However, RoTTV1 RNA was not detected in these tissue

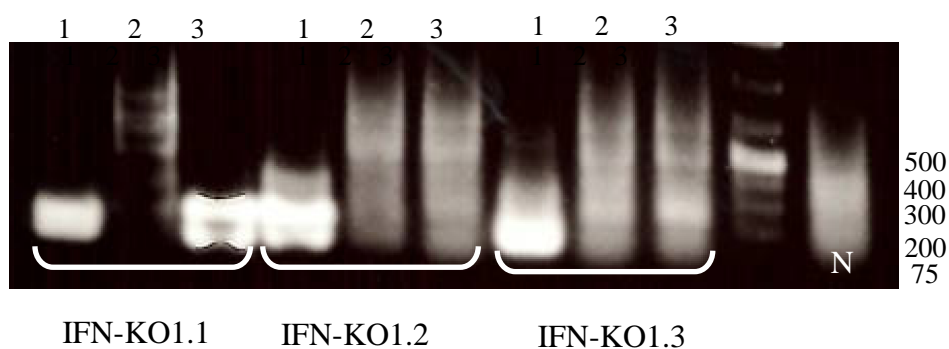


Figure 6.2.3. Screening PCR of Type I IFN receptor KO mice inoculated RoTTV1 concatemerised DNA.

N: negative control, IFN-KO: Type I IFN receptor KO mouse

Lane1: lung, Lane2: liver, Lane3: spleen

RoTTV1 DNA was observed in lung of all mice and also detected in spleen of mouse 1.

6.3. TTV infection using cell culture supernatant in wood mouse

Experimental protocol

Section 6.2 showed RoTTV1 DNA was detectable at two month post-infection but RoTTV1 RNA and robust RoTTV1 infection was not observed using *in vivo* transfection of the concatemerised DNA. In chapter V, RoTTV1 was propagated in BHK-21 cells, therefore this virus was used to infect mice. Female wood mice aged 80-90 days were infected intranasally with 10^5 copies of generated virus. For each of 3 time points (2 weeks, 4 weeks and more) four mice were infected. As the negative control, fresh Glasgow Minimum Essential Medium (Invitrogen) was also inoculated intranasally into two female mice aged 80-90 days old per time point.

DNA was extracted from the lung and spleen from 4 infected and 2 negative control wood mice two weeks post-infection and PCR was performed by using RoTTV1 species specific primers. RoTTV1 was not observed in lung and spleen from four infected mice and RNA was also negative by RT-PCR. The same result was also observed in the wood mice 4 weeks post-infection. Mice from the final time point are under study.

6.4. Discussion

Experimental design based primarily on TTSuV paper

The *in vivo* experiment was designed based on a published study of TTSuV2 infection (Huang *et al.*, 2012). Concatemerised and single copies of TTSuV2 DNA were directly injected into lymph node and muscle of 40 days old piglets and TTSuV2 replication was observed.

Age

As the previous study used young piglets (Huang *et al.*, 2012), young wood mice were used for RoTTV1 infection in this study. Young individuals have relatively less developed immunity and are growing rapidly and therefore likely to have more actively replicating cells. TTV is predicted to use the host cellular replication machinery and infect the highly replicating cells (Kakkola *et al.*, 2007). RoTTV1 is more likely to infect these young and actively replicating cells. One year old mice were used for *in vivo* infection in a pilot study but no virus DNA was detectable in the study (Data not shown). Therefore, the young mice used in present study were hoped to be suitable for experimental RoTTV1 infections. However, it is possible that infection of even younger mice may be necessary for the robust virus replication.

Concatemerised DNA/cultured virus

Concatemerised DNA was also used in chapter V for *in vitro* study. The advantage of the dimerised genomes is that a continuous complete genome sequence from any given point is present and uninterrupted by vector sequence (Fig. 2.15.7.1.). In the previous study of TTSuV2 (Huang *et al.*, 2012), cocatermerised TTSuV2 DNA and single copies of TTSuV2 DNA were injected into the piglets. Accordingly, concatemerised RoTTV1 DNA was also used for *in vivo* transfection.

In chapter V, cultured viruses were generated from BHK-21 cells and the viruses containing culture supernatant used to infect fresh BHK-21 *in vitro*. Therefore, culture supernatant was also used for the inoculation to more accurately mimic natural infection.

Route

Due to the small size of *Apodemus sylvaticus*, the infectious agent cannot be easily injected directly into the lymph nodes. Also, we have seen RNA in lung tissues frequently (section 4.2) and it was predicted that lung tissue is one of the important replication sites for RoTTV1. Thus, infectious agents were inoculated into wood mice and IFN receptor KO mice intranasally.

Naked DNA injection has sometimes been used for virus infection such as murine hepatitis B virus and hepatitis delta virus (Chang *et al.*, 2001; Chang *et al.*, 2003). In addition to that, in the present study, PEI transfection reagent was used for *in vivo* transfection. PEI is used frequently for *in vivo* and *in vitro* transfection and it has been used to successfully deliver DNA efficiently in a number of systems (Behnam *et al.*, 2013; Huang *et al.*, 2013). PEI would support penetration of the cell membrane by viral DNA and increase the chance for the successful transfection.

Initial experiment result

In this study, RoTTV-1 DNA was detectable even one month post-infection and it is indicated that PEI successfully functioned as an *in vivo* transfection reagent in present study. However, no RNA was observed in these tissues.

RoTTV1 distributions were different among the individuals, and mouse 4 showed high viral copy number in the lung and liver only, but RoTTV1 DNA was detectable in various tissues in mouse 1.2. Human TTV DNA has been detected in all tissues and serum (Reviewed by Hino and Miyata., 2007) and it is predicted that, if replicating, RoTTV1 DNA would also be observed in the various tissues.

There are several interpretations of this experiment, especially because of the lack of RNA detection in virus DNA positive tissues. RoTTV1 replication might be low level and not to sufficient detect RNA. Therefore, a longer experimental time may be required to produce the detectable RNA. This is also discussed in the second experiment. Also, the main replication site

may be in tissues and other than those that we screened for RNA detection.

Based on a previous study of plasmid persistence following *in vivo* transfection, the level of DNA shown in this initial experiment was high and these DNA were unlikely to be due to the remaining redistributed input DNA (Dr. Gerry McLachlan, personal communication). Therefore, we need to confirm the viral replication by using 2 methods, DNase I treatment and vector specific qPCR. DNase I digests only the double-stranded DNA, and input plasmid DNA and replicated TTV would be distinguishable by using this enzyme. Furthermore, qPCR could be used to compare total TTV DNA copy number and input plasmid copy number by using TTV and vector sequence primers and probes. In future work, this will have to be investigated to confirm the virus replication.

Second experiment

In the second study, it was expected that the first experiment would be confirmed but the *in vivo* transfection did not appear to be as efficient compared to initial experiment. The transfection reagent and concatemerised DNA were older and may be less efficient, but these reagents are known to be stable materials. Another possible reason is that the wood mice (*Apodemus sylvaticus*) are sensitive to anesthesia and there were problems with anesthesia in the second experiment. Wood mice are not widely used as experimental animals and more work may be required to optimize anaesthesia conditions. Because of these problems, it is possible that the amount of DNA delivered into the animals in the second experiment was too low. This low amount of RoTTV1 DNA load would hamper establishment of the virus infection and it may take a much longer to establish the detectable infection or host immune system could eradicate the virus. It is not clear why the efficiency of PEI transfection decreased in second infection and further study is needed to establish a robust procedure for transfection of TTV DNA into wood mice.

RoTTV1 concatemerised DNA was cleared from 3 mice one month post-transfection but

RoTTV1 DNA was detected from all 4 mice two months post-transfection. We hypothesise that RoTTV1 replicates slowly and therefore it is difficult to detect RoTTV RNA in early phase of infection. It has also been suggested that human TTV replicates slowly (Okamoto *et al.*, 1999; Biagini *et al.*, 1999; Khudyakov *et al.*, 2000). Active mutation occurs in fast replicating viruses and highly replicating viruses tend to show the high genetic diversity (section 1.2.), but TTV might take long time to achieve their high genetic diversity (Biagini *et al.*, 1999; Khudyakov *et al.*, 2000).

A second hypothesis is that RoTTV-1 primary infection may occur preferentially in females. In this study, all RoTTV1 DNA positive mice from the 2 *in vivo* transfection experiments were female except 1 male wood mouse and 3 male Type I IFN receptor KO mice. It may indicate that female individuals are more susceptible to TTV than males. However, there is no report that TTV prevalence or susceptibility of female individuals is higher than those of males and further experiments in the current system would be required to demonstrate a correlation.

We also hypothesized that TTV would infect Type I IFN receptor KO mice and RoTTV1 DNA was detectable in lung and spleen one month post infection. However, further studies are required to determine if RoTTV1 infection can be established in Type I IFN receptor KO mice. Type I IFN receptor KO mice are *Mus musculus* and in chapter III, RoTTV infection was not observed only in *Mus musculus*. In chapter V, Type I IFN receptor KO MEF cells did not support the continuous replication of RoTTV1 and it may indicate that the cells of *Mus musculus* may lack the function to reproduce RoTTV.

In addition to the other possibilities, the wood/lab mice used in this study might not be young enough for RoTTV infection. Young animals show the high replication (discussed above) and have not developed their robust immune system. Also, human TTV is also predicted to infect the hosts in early stage of life (Ninomiya *et al.*, 2008). Accordingly, it is predicted that younger individuals are more likely to become infected with RoTTV. In this study, 30 days-old wood/lab

mice were used for the infection. In the lab, mice were weaned at 30 days of age and 30 days piglets were used for the previous pig *in vivo* experiment (Huang *et al.*, 2012). However, to compare the life span of pigs and mice, 30 days old mice are relatively much older than those of pigs. Therefore, younger mice might be required for RoTTV1 infection.

In chapter V, RoTTV1 replication was observed in BHK-21 cells and RoTTV1 in culture supernatant was inoculated into the wood mice. However, virus replication was not found in these wood mice. Wood mice have been found to have stronger immune responses including a stronger interferon response and natural killer (NK) cell activity than lab mice in Central European encephalitis(CEE) virus and parasite infection models (Kopecky *et al.*, 1991; Francois *et al.*, 2010; Jackson *et al.*, 2009) (section 3.8.1.). Despite these robust immune responses, a high infectious prevalence of RoTTV in wood mice is seen. This may indicate that intranasal infection might not be a suitable infectious route for RoTTV1. Similar difficulty of virus infection was also observed in murine gammaherpesvirus-68 (MHV-68). For human gamma herpes virus such as EBV and KSHV, virus transmission routes have been suggested including horizontal transmission by saliva (Niederman *et al.*, 1976; Hadinoto *et al.*, 2009), vertical transmission by uterine cervix (Sixbey *et al.*, 1986; Silver *et al.*, 2011), and sexual transmission by male genital tract (Israele *et al.*, 1991; Naher *et al.*, 1992). However, for MHV-68 infection, the main infectious route is still not known (Francois *et al.*, 2013). Horizontal transmission of MHV-68 has been studied for 30 years but transmission under laboratory conditions has not been observed (Barton *et al.*, 2011). However, recently, one paper showed the sexual transmission in lab mice (Francois *et al.*, 2013). Two hypotheses have been suggested to explain the results. Firstly, mice cannot perform the physiological behaviors such as scent-marking and male fighting in conventional animal caging. Secondly, MHV-68 life cycle has been well-studied but unexplored infectious routes such as the sexual transmission could influence of the results (Francois *et al.*, 2013). Therefore, future and ongoing studies may have to consider the other infectious routes, virus copy number, and physiology of wood mice for RoTTV1 infection.

In conclusion, RoTTV1 was inoculated intranasally by using two agents, *in vivo* transfection and isolated RoTTV1 infection. In *in vivo* transfection, potential production of RoTTV1 DNA was observed in wood mice but more work is required to establish a robust infection protocol for RoTTV1. Also, new experimental design including physiological knowledge of wood mice and new infectious routes may be needed for the inoculation of isolated viruses. Further study has to be performed.

CHAPTER VII. Conclusion

The objective of the current study was to investigate the possibility of rodent TTV as a human TTV animal model. Rodent TTV (RoTTV) was found in wild wood mice by using RCA in our laboratory and RoTTV represents a powerful tool for the study of characterisation of virus nature, transcripts profile identification, protein identification, cell culture system and pathogenesis *in vivo*.

Characterisation of rodent torque teno virus in wild rodent population

At the start of study, RoTTV was characterised by using the wild rodent samples. It was revealed that RoTTV genomic structure was similar to that of human and other animal anelloviruses. TTV is a small single-stranded DNA virus and the genome size is 2.2kb in RoTTV-1 and 2.5kb in RoTTV-2. RoTTV is smaller than human TTMV which is the smallest size of human anellovirus, but the genomic structures are similar to those of human and other animal anelloviruses. The UTR which is relatively conserved in all anellovirus genomes was also observed in RoTTV-1 and RoTTV-2 and RoTTV ORF2 was located upstream of ORF1 as in other anelloviruses.

RoTTV showed a high infectious prevalence in the wild rodent population, especially in wood mice (*Apodemus sylvaticus*) but was absent from *Mus musculus*. Furthermore, 2 virus species containing multiple genotypes were found in RoTTV and the different genotypes of viruses were often present in a single individual. This high infectious prevalence and the multiple infection in single individuals are both characteristic of human TTV infection (Bendinelli *et al.*, 2001; Moen *et al.*, 2002; Maggi *et al.*, 2005; Biagini *et al.*, 2006a; Biagini *et al.*, 2006b; Ninomiya *et al.*, 2008, Maggie and Bendinelli. 2010).

The genomic structure, genomic diversity and high infectious prevalence of RoTTV are similar to human and other animal anelloviruses and this suggests that RoTTV could be an ideal animal model for TTV infection.

Transcript identification and protein expression of rodent torque teno virus

The transcriptional profile of anelloviruses has only been studied for human and porcine TTVs (TTSuV) (Martinez-Guino *et al.*, 2011). It is important to understand the transcriptional profile and protein function for the study of pathogenesis of TTVs and other anellovirus. In this study, 2 transcripts coding 4 predicted proteins and 5 transcripts coding 9 predicted proteins were observed from RoTTV-1 and RoTTV-2, respectively. Based on the transcriptional profile of RoTTV-1, protein expression was performed in BHK-21 cells and ORF2, ORF2-3, ORF1-4 showed the different expression pattern in BHK-21 cells and these proteins were detected by western blot. However, full length ORF1 was not detected by western blot although ORF1 showed the distinctive pattern of protein expression in BHK-21 cells. Similar difficulties in detection of human and porcine TTV ORF1 proteins were reported (Qiu *et al.*, 2005; Martinez-Guino *et al.*, 2011). However, one human TTV report succeeded in detection of full length ORF1 protein and it was concluded that the use of different plasmids and/or protein tags may allow the detection of ORF1 by western blot (Muller *et al.*, 2008). Therefore, RoTTV-1 ORF1 protein expressed by different plasmids might be detected by western blot.

RoTTV-1 ORF2 and ORF2-3 showed similar lower bands and these smaller bands were predicted to be the result of RNA splicing or cleavage after translation. Accordingly, RT-PCR should be carried out by using RNA extracted from ORF2 and ORF2-3 transfected BHK-21 cells to confirm RNA splicing. No predicted cleavage/splicing could not be found in these proteins. The products that were seen in the present study would have to be protein-sequenced to identify the protein and confirm whether the lower bands from ORF2 and ORF2-3 are identical or not. Further study is required to understand the function of these proteins to study their role in the pathogenesis of RoTTV.

Torque teno virus replication *in vitro*

The lack of a good system for *in vitro* TTV replication has been a major problem for research in the field. In the wild, RoTTV is presented in a relatively wide variety of rodent species and the

several cell cultures were prepared for RoTTV replication. In this study, RoTTV-1 showed continuous and stable replication in BHK-21 cells and it is the first time a robust anellovirus cell culture system has been described. For future studies, characterising the replicated virus may be required. In a recent study, TTV transfected 293TT cells reproduced subviral molecules of TTV (μ TTV) and their genome sizes were smaller than the original genome of human TTV (de Villiers *et al.*, 2011). In human studies, intragenomic rearrangements of TTV have also been reported (Leppik *et al.*, 2007) and RoTTV also can be sequenced in each time point to compare the mutation rate and genomic structure. This result would be the starting point to understand of the virus-cell interaction and molecular and biochemical mechanism of TTV infection.

Furthermore, RoTTV-1 replicated efficiently in young passage BHK-21 cells compared to old BHK-21 cells. Future studies will require strict conditions to study the virus replication including the same batch of cells and long term experiments to show the clear difference between old and young BHK-21 cells.

TTV virus propagation was observed in BHK-21 cell culture. RoTTV-1 RNA was detected in the fresh BHK-21 cells cultured by using RoTTV-1 containing culture supernatant. This is also the first time anellovirus propagation *in vitro* has been clearly demonstrated. For further confirmation of virus replication, electron microscopy would be used for finding RoTTV.

***In vivo* experiment of Torque teno virus infection in wood mice and type I IFN KO mice**

In the present study, *in vivo* transfection of concatemerised DNA was used as a potential infectious method. Although RoTTV-1 DNA was cleared one month post-transfection in some cases, detectable RoTTV-1 was observed at two months post-infection. In a previous study TTSuV2 concatemerised DNA was directly injected into the lymph node and muscle and the virus replication was observed. In the present study, we can suggest a new approach which is more likely to deliver TTV genomic material into the host cells by using PEI transfection reagent.

For the future study, RoTTV-1 RNA and systemic infection are needed to show the robust infection. For the *in vivo* transfection, we believe that further experiments with longer infection period may be needed to detect robust virus replication.

An infection experiment was also performed by using the supernatant of culture fluid. The culture fluid was inoculated intranasally but no replication was observed. TTVs are predicted to use the faecal-oral route for the transmission and RoTTV-1 RNA was detected in the lung and bone marrow. Accordingly, it was presumed that the intranasal infection would be the best way to apply the virus to the lung and gastrointestinal tract. However, sometimes, the virus infection requires the complex mechanism like murine gamma herpes virus (Barton *et al.*, 2011) and RoTTV-1 infection may be achieved depending on the behavior of rodents such as scent-marking and male fighting. This can also explain why wood mice showed the high infectious prevalence compared to the other rodent species such as field voles and bank voles. Further study is required to understand the natural infection of RoTTV in wild rodents.

In conclusion, we characterised RoTTV; virus epidemiology in wild rodent species, genetic diversity, RNA localisation, transcriptional profile, protein expression *in vitro*, and protein localisation in the cultured cells in this study. Also, a robust cell culture system was established for the continuous replication of RoTTV-1. Finally, as a new approach of TTV infectious experiment, PEI *in vivo* transfection could be suggested for *in vivo* study of RoTTV. This basic knowledge of RoTTV would provide a starting point for future experiments and it is predicted that TTV pathogenesis, host-virus interaction, and immunology can be studied by using the mouse animal model.

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